ROLE OF CYTOCHROME P450 ENZYMES IN ACUTE MYELOID LEUKEMIA BONE MARROW MICROENVIRONMENT

by
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ABSTRCT

My studies focused on identifying cell-extrinsic mechanisms of drug resistance of Acute Myeloid Leukemia (AML) in order to develop improved therapies. In Chapter 2, we discovered human bone marrow (BM) mesenchymal stromal cells (MSCs) highly express the primary retinoid inactivating enzyme, Cytochrome P450 26 (CYP26). CYP26 protects both APL and non-APL AML cells from the pro-differentiation effects of all-rans retinoic acid (ATRA). Inhibition of CYP26 rescued ATRA levels and AML cell sensitivity in the presence of BM stroma. Our data suggest that stromal CYP26 activity creates retinoid low sanctuaries in the BM that protect AML cells from systemic ATRA therapy. Inhibiting or bypassing CYP26 provides new opportunities to expand the clinical activity of ATRA in both APL and non-APL AML. We also discovered that BM MSCs express a wide variety of CYPs and other drug metabolizing enzymes, similar to hepatocytes. Importantly, CYP3A4, which is responsible for inactivating half of currently available chemotherapeutics, is highly expressed in the BM MSCs and protected leukemia stem cells (LSCs) and multiple myeloma (MM) CSCs. Inhibiting CYP3A4 overcame the stroma's chemoprotective capacity. In Chapter 3, we found that the CYP3A4 and cytidine deaminase (CDA) expression levels of BM MSCs are down-regulated by AML cells. More so, AML chemotherapy drugs cytarabine (ara-C), daunorubicin and etoposide (VP-16), induce CYP3A4 and CDA levels in MSCs as well as increase stromal protective effect. Understanding how CYPs levels vary in AML BM during treatment provides a basis for combining a stromal CYP3A4 inhibitor with chemotherapy to improve the elimination of minimal residual disease (MRD). These studies thus identify mechanisms of differentiation therapy and chemotherapy drug

resistance in AML, and our preclinical data suggest some potential therapeutic applications.

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Introduction

1.1 Bone Marrow Microenvironment

1.1.1 Overview

Hematopoietic stem cells (HSCs) are essential to preserve hematological homeostasis and regeneration, and are the only cells capable of giving rise to all blood cell lineages [1, 2]. The bone marrow (BM) microenvironment is essential for HSCs to maintain lifelong blood production, by balancing its self-renewal and differentiation. Within the BM microenvironment, HSCs communicate with surrounding cells through cell-cell contacts and soluble factors. These interactions are essential to maintain HSCs by regulating their quiescence, self-renewal and differentiation [3, 4]. Analogously to normal stem cells, cancer stem cells (CSCs) reside in specific niches that maintain their tumor initiation capacity and protect them from threats posed by the immune system and chemotherapy [5, 6].

A better understanding of hematopoiesis and cancer cell biology has resulted in rapid therapeutic advances, leading to increased response rates and prolonged survivals. However, these advances have not generally translated to more cures. Increasing evidence suggests that rare populations of CSCs, characterized for their ability to self-renew, differentiate and survive chemotherapy, are significant contributors to minimal residual disease (MRD) and relapse [7-9]. Research has traditionally focused on cell-intrinsic mechanisms, such as quiescence or genetic heterogeneity, to understand the survival advantage of CSCs during chemotherapy [10]. However, over the past few years, the mechanism by which the microenvironment contributes to chemotherapy resistance of this malignant population has been an area of active research [11, 12]. Disrupting the

communication with their niche holds potential for eliminating the malignant clones that reside at the top of the cancer hierarchy.

Many patients with AML are able to achieve complete remissions (CRs) with standard chemotherapy. However, CRs do not mean cures for most of these patients, and the majority will eventually relapse and die of their disease. The cancer stem cell (CSC) model provides a reasonable explanation for this gap between remission and cure. The current therapies often kill only the differentiated bulk of the cancer, but the more resistant CSCs then regenerate the disease. Besides of the important cell intrinsic mechanisms of CSC drug resistance, there is much evidence that the BM microenvironment also plays an important role in CSC drug resistance. However, the mechanisms of BM microenvironment mediated drug resistance were poorly understood.

Definitive evidence for the existence of stem cells was provided first in the hematopoietic system, when Till and McCulloch reported the presence of hematopoietic colonies in the spleen of irradiated mice after transplantation with BM cells [1]. And it was also in the hematopoietic system where the term “stem cell niche” was described for the first time, after Schofield found that stem cells from the bone marrow had the ability to reconstitute the hematopoietic system indefinitely, while spleen-derived stem cells previously reported by Till and McCulloch did not have this ability [13]. He suggested the existence of a distinctive niche within the BM essential to maintain a stem cell phenotype, by preventing the maturation of these cells.

Analogous to the role of normal stem cells in maintaining lifelong tissue homeostasis, evidence gathered over the past two decades suggests that a rare population of cancer cells with the ability to self-renew and differentiate is responsible for giving rise and propagating cancer [7-9]. Strong support for the CSC model was provided by Lapidot *et al* who observed that only a rare subset of leukemia cells, compromising less than 0.01% of the total population, were able to engraft and produce acute myeloid leukemia (AML) in mice [8]. Later studies further supported this hypothesis in AML [14, 15], which appears to organized in a hierarchical manner similar to normal hematopoiesis, with primitive CD34+ CD38- stem cells as the target of malignant transformation but the bulk of disease representing differentiated progeny. As such, leukemia stem cells (LSCs) can be considered true stem cells with the ability to: 1) differentiate into the bulk differentiated leukemia cells; and 2) self-renewal as evidenced by their ability to engraft immunodeficient mice upon serial transplantation.

Although LSCs were initially described in AML as a relatively homogenous CD34+ CD38- population, it is now known that they are phenotypically as well as genetically heterogeneous [16, 17]. With a few exceptions, such as TKIs inhibitors in CML, this cellular and molecular heterogeneity has complicated the development of targeted therapies for specific mutations or markers. For this reason, treatment of hematologic malignancies has relied on the so-called "traditional chemotherapy" which targets rapid proliferative cells. These classic cytotoxic agents often leave behind CSCs, which unlike the bulk of the malignant cells but similar to normal HSCs, are characterized by a dormant or quiescent state in part induced by the microenvironment [11, 12]. Thus, better

understanding the biology of the CSCs microenvironment could allow novel anti-cancer therapies aimed at disrupting its influence on CSCs.

1.1.2 The hematopoietic stem cell niche

Bone marrow is a complex organ containing many different hematopoietic and non-hematopoietic cell types. Bone marrow is surrounded by a shell of vascularized and innervated bone **(Figure 1.1).** The HSCs reside primarily within bone marrow during adulthood. The hematopoietic niche can be broadly subdivided into a perivascular area, located close to the central vein of the BM, and an endosteal area, located close to the internal lining of the bone. Both areas harbor a variety of stromal cells, including osteoblasts, fibroblasts, adipocytes, endothelial cells and mesenchymal stem cells (MSCs) [3, 4] **(Figure 1.2).**

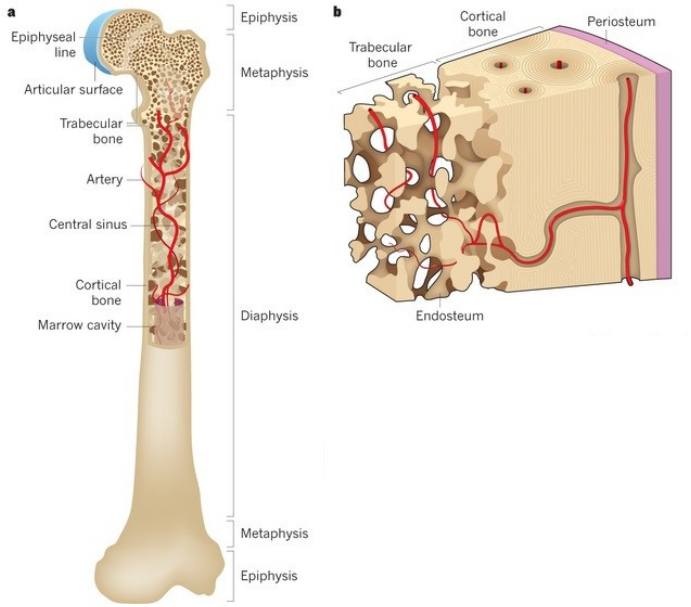

Figure 1.1. Bone marrow anatomy

a. Minute projections of bone (trabeculae) are found throughout the metaphysis such that many cells in this region are close to bone surface. b. The interface of bone and bone marrow is known as the endosteum, which is covered by bone-lining cells that include bone-forming osteoblasts and bone-resorbing osteoclasts. Arteries carry oxygen, nutrients, and growth factors into the bone marrow, before feeding into sinusoids, which coalesce as a central sinus to form the venous circulation. Sinusoids are specialized venuoles that form a reticular network of fenestrated vessels that allow cells to pass in and out of circulation. There is a particularly rich supply of arterioles as well as sinusoids near the endosteum. (Modified from Morrison, S.J. and Scadden, D.T. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014; 505: 327–334)

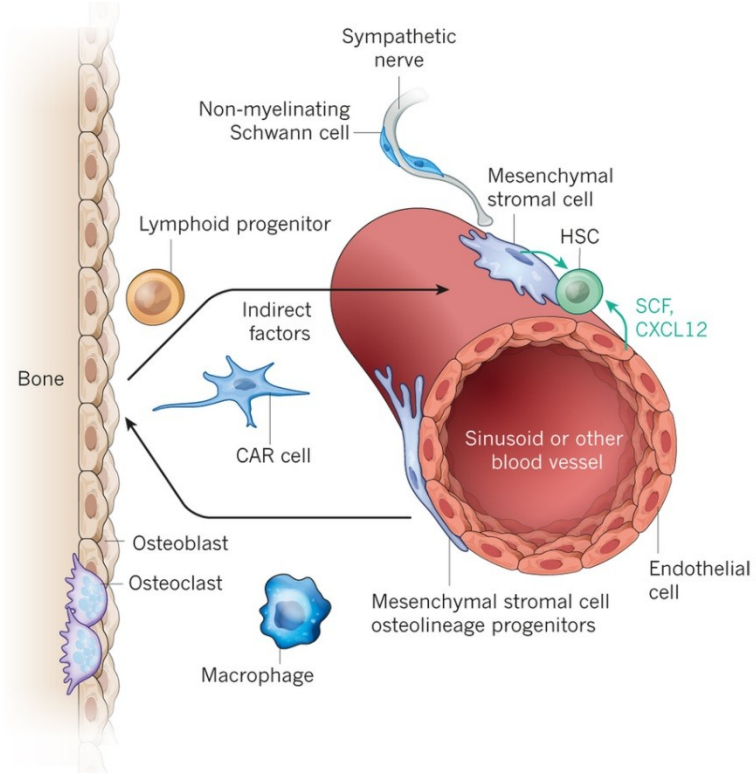

Figure 1.2. Haematopoietic stem cells (HSCs) and restricted haematopoietic progenitors occupy distinct niches in the bone marrow. (from Morrison, S.J. and Scadden, D.T. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014; 505: 327–334)

The majority of HSCs have a perivascular location, and 60-67% are located within 20 μm of the sinusoidal endothelium, most of them in the endosteal region of the bone [18-21]. Different cell types and the cytokines, adhesion molecules or extracellular matrix proteins are associated with this regional localization. MSCs, defined by their trilineage (adipocytes, osteo- and chondro-lineage) differentiation potential and expression of Nestin and LepR, are in close contact with sinusoidal endothelium [22, 23]. Moreover, MSCs have the highest expression of genes associated with HSCs maintenance, including CXCL12, stem cell factor (SCF or kit ligand) or angiopoietin-1, and their depletion significantly compromises hematopoiesis [21]. Endothelial cells (ECs) surrounding arterioles and sinusoids are also part of the BM niche. Similar to the role of MSCs, expression of SCF and CXCL12 by ECs promotes HSCs maintenance and self-renewal [19, 24].

Osteoblasts were the first component of the BM niche found to regulate hematopoiesis. Initial studies showed that osteoblasts supported the growth of HSCs in vitro [25, 26], followed by in vivo experiments demonstrating that osteoblastic activation or expansion resulted in increased numbers of HSCs and increased survival of irradiated mice after BM transplantation. Further, depletion of osteoblasts was associated with severe loss of HSCs from the BM, with extra-medullary hematopoiesis [27-29].

Recent studies have suggested that not only stromal but also hematopoietic components are essential for HSCs maintenance. HSCs are non-randomly located close to megakaryocytes, and their depletion results in loss of HSCs quiescence, compromising

repopulation ability [30, 31]. In addition, macrophages are essential for erythropoiesis and recovery from hemolytic anemia and acute blood loss [32].

In summary, different cellular components of the BM niche may have either direct or indirect roles in hematopoiesis. Based on studies demonstrating that the majority of HSCs localize close to blood vessels, and that deletion of HSCs-retaining genes from ECs and MSCs, but not from osteoblasts, compromises hematopoietic recovery after transplantation, some have concluded that the niche is perivascular [19, 21].

1.1.3 Leukemia stem cell (LSC) niche

The facts that BM is a common metastatic site for solid tumors, and that leukemia cells migrate early to bone marrow spaces throughout the body, before metastasizing to other tissues, suggests that the BM provides a unique niche for malignant cells. Moreover, the BM serves as sanctuary for MRD, not only in hematologic malignancies, but also in solid tumors [11, 33, 34]. When examined in long bones, such as the femur, engraftment of LSCs concentrates in the endosteal regions, while their differentiated progeny expands, migrates and packs in the central region of the bone marrow [35]. After treatment with chemotherapy, apoptosis is only evident in the central BM cavity, while quiescent leukemic cells within the endosteum are spared, eventually causing relapse [36].

Many of the mechanisms by which the BM microenvironment maintains HSCs are co-opted by CSCs to perpetuate cancer, promoting a balance of self-renewal, differentiation and proliferation. Within the endosteal region, leukemia cells bind to sinusoidal

perivascular cells in an identical fashion to normal HSCs [37, 38]. This interaction is facilitated by perivascular SCF-1 (also called CXCL12), which binds to CXCR4 on malignant cells. LSCs appear to be selectively beneficiated by these physical contacts, since expression of CXCR4 on CD34+ leukemic blasts, but not on the bulk of leukemia cells, is correlated with worse prognosis [39].

Similarly to CXCR4-CXCL12, the interaction between leukemic VLA-4 and stromal fibronectin is also critical for maintaining LSCs [40]. Consistent with this, patients whose AML blasts are negative for this adhesion molecule have significantly increased survival compared to VLA-4 positive patients [41]. Multiple myeloma (MM) cells also employ adhesion molecules to home and survive within the bone marrow niche [42, 43].

Therapeutic strategies to overcome microenvironmental chemoprotection have focused on displacement of malignant cells from the BM niche by targeting adhesion molecules or chemokines. Preclinical studies have demonstrated that inhibition of the CXCR4-CXCR12 interaction impairs engraftment of leukemic cells, which remain in the circulation instead of homing to the BM [44-46]. Similarly, blocking VLA-4 results in a dramatic increase of AML cells in the peripheral circulation, and combination of this antibody with ara-C increases improved AML kill in vitro [40]. Expression of CD44, another adhesion molecule expressed on malignant cells, is also associated with maintenance of a CSC phenotype and drug resistance, and its inhibition reduces the size of the CSC compartment and enhances chemotherapy efficacy in preclinical models of AML, CML and MM [47-49]. However, clinical benefit from mobilizing cancer cells

from the bone marrow has not been demonstrated in the clinic. Thus, the molecular machinery directly responsible for chemoprotection within the BM niche is incompletely understood.

1.2 Cytochrome P450 Enzymes (CYPs) and drug metabolism

CYPs are one of the most important enzyme families involved in the metabolism of xenobiotics. CYPs comprise many isoforms, which catalyze a wide variety of reactions. Five of the isoforms, 1A2, 2C9, 2C19, 2D6 and 3A4, metabolize about 90% of the marketed drug compounds [50]. Most CYPs are expressed in the liver, including CYP3A4, which as the most abundant in human liver accounts for about 30% of total hepatic CYPs [51]. CYPs are also present in nearly all tissues, especially those exposed to outside foreign toxins, such as the small intestine, kidney, and lung.

CYP expression has also been found in a variety of human tumors, including the bladder, breast, colon, esophagus, kidney, lung, ovary, prostate, stomach [52, 53]. The CYPs are important for both cytotoxic bioactivation, as well as drug metabolism. CYP3A4 plays an important role in the metabolism of many anticancer agents, including the topoisomerase inhibitor etoposide, which is one of the most important agents in the treatment of AML [54], the taxanes paclitaxel and docetaxel, which are used in the treatment of several tumor types including breast, ovarian, head & neck and lung cancer [55, 56], and irinotecan, an important drug in colorectal cancer treatment [57].

1.2.1 Retinoid acid and CYP26

Vitamin A (retinol) plays a critical role regulating the differentiation, growth, and migration of many cell types. Classically, the term vitamin A is used to describe retinol and retinyl esters **(Figure 1.3)**, including retinyl palmitate, which are the main dietary forms of vitamin A obtained from animal products [58, 59]. However, retinol and retinyl esters require oxidation by several enzymes to form the biologically active metabolite, retinoic acid (RA), the main compound responsible for the biological activity of vitamin A.

The retinoid pathway is summarized in **Figure 1.4**. The two naturally-occurring isomers of RA, all-trans-RA (ATRA) and 9-cis-RA, are produced by tissue-specific metabolism of Vitamin A (retinol) via intracellular alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH1) [60]. The biologic effects of RA are mediated by binding to two families of nuclear hormone receptors, the RA receptor (RAR) and the retinoid X receptor (RXR) families [61]. Each family consists of three isotypes (α, β, and γ) that function as RAR/RXR heterodimers.

The inactivation of ATRA is predominantly by cytochrome P450 family 26 enzymes (CYP26) [62], while some other CYPs including human CYP3A4, CYP2C8 and rat CYP2C22 also play a lesser role in this process [63-65]. It is notable that, the affinity of ATRA to CYP26 is about 1000- fold higher than it to other CYPs, and the overall intrinsic clearance of ATRA by CYP26 enzymes is 1000- to 10,000-fold higher than other CYPs [66]. Thus, even in tissues such as the human liver with high expression

levels of CYP3A4 and CYP2C8, the CYP26s, even are expressed at low levels, are still the main contributors to ATRA clearance [62, 66, 67]. CYP-mediated inactivation of ATRA is through oxidization at the four-position of the β-ionone ring of ATRA and generate 4-OH-RA. The CYPs can also generate several other metabolites from ATRA, including 18-OH-RA and 16-OH-RA [67, 68].

CYP26 is a family of three highly conserved isoenzymes, CYP26A1, CYP26B1, and CYP26C1. They only share 40-50% sequence similarity in any species [62]. CYP26A1 and CYP26B1 are the primary members, inactivating both vitamin A and RA; CYP26B1 is the most highly conserved among these three [62, 69].

CYP26A1 and CYP26B1 mRNA and protein are found in multiple extrahepatic human tissues [68, 70]. The RA concentrations in certain tissues are determined by RA synthesis and clearance locally, instead of the systemic RA concentrations. CYP26 has been shown to establish a barrier for RA delivery from circulation to specific organs, such as the testes, pancreas, and spleen [71-73]. Therefore, the extrahepatic expression and activity of CYP26 enzymes in specific tissues and cell types play critically important roles in tissue-specific RA regulation and defining the relationship between RA and biological outcomes in certain tissues.

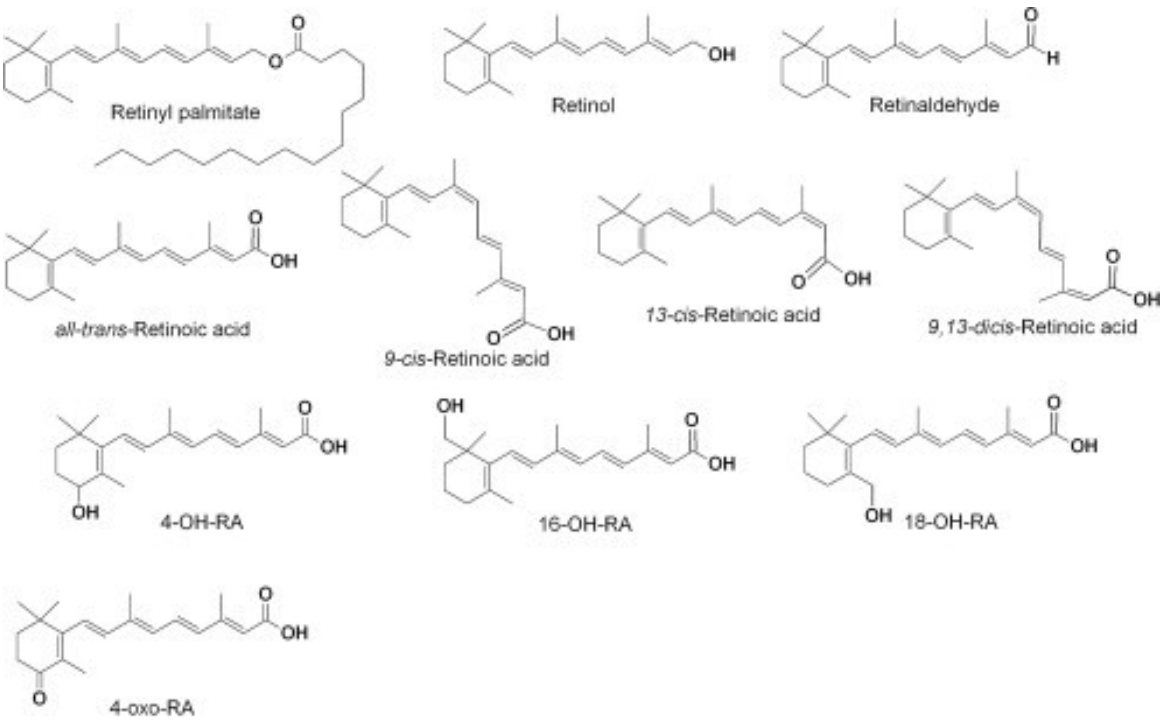

Figure 1.3. Chemical structures of retinyl palmitate, retinol, retinaldehyde, and the retinoic acid isomers and metabolites. (from Stevison, F et al. Role of Retinoic Acid-Metabolizing Cytochrome P450s, CYP26, in inflammation and Cancer. *Adv Pharmacol.* 2015;74:373-412.)

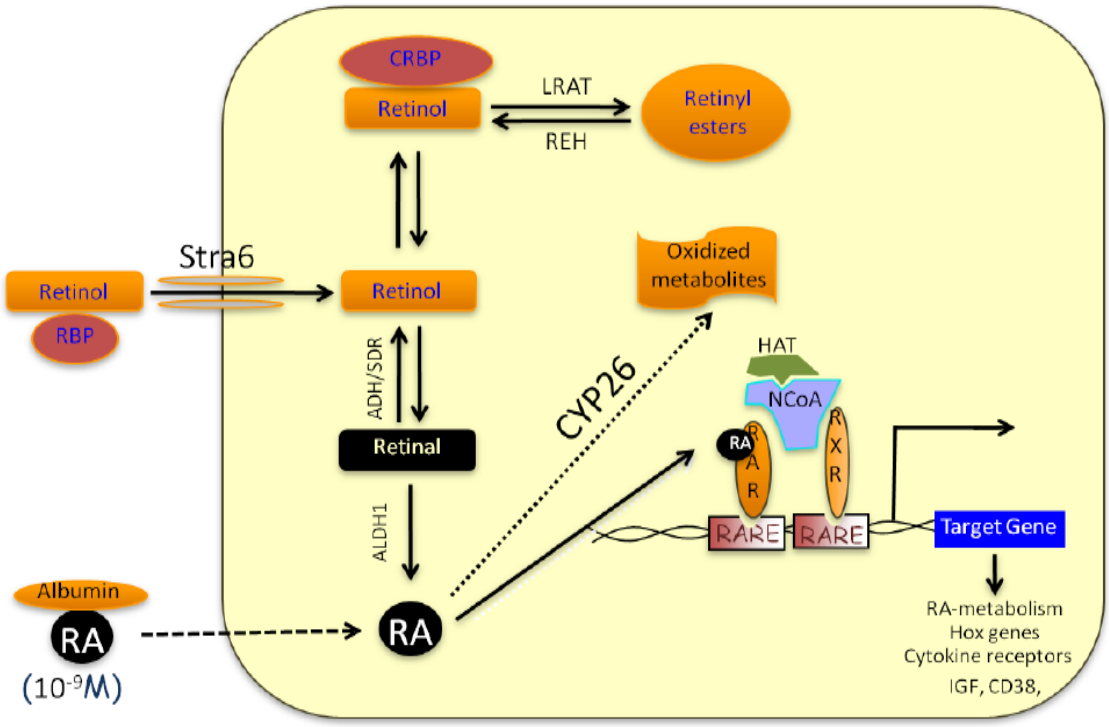

Figure 1.4. Retinoid pathway. Vitamin A (retinol) is metabolized to retinoic acid (RA) via alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH1). Vitamin A and RA are inactivated by CYP26B1.

1.2.2 CYP3A4 and anticancer drugs

CYP3A4 is involved in the metabolic clearance of most commonly used anticancer drugs, including: the taxanes, docetaxel and paclitaxel; the topoisomerase I inhibitors, irinotecan and topotecan; the topoisomerase II inhibitors, etoposide and teniposide; the vinca alkaloids, vincristine, vinblastine, vindesine and vinorelbine; hormonal therapies including exemestane, tamoxifen, fulvestrant and toremifene; and newer molecular targeted agents such as gefitinib and imatinib **(Table 1)** [74, 75]. In the context of cancer chemotherapy, most of the drugs have considerable dose-related toxicity. Therefore, relatively small differences in the disposition of anticancer drugs in local tissues can lead to major differences in both toxicity and efficacy. Understanding the mechanism of the anticancer drugs metabolism and drug resistance is very important to improving patient outcomes.

Table 1. Examples of anticancer drugs exhibiting significant CYP3A-mediated metabolism.

Drug class	Anticancer drug
Taxanes	Docetaxel
	Paclitaxel
Topoisomerase I inhibitors	Irinotecan
	Topotecan
Topoisomerase II inhibitors	Etoposide
	Teniposide
	Mitozantrone
Vinca alkaloids	Vincristine
	Vinblastine
	Vindesine
	Vinorelbine
Alkylating agents	Cyclophosphamide
	Ifosfamide
	Thiotepa
Hormonal agents	Tamoxifen
	Exemestane
	Letrozole
Molecularly targeted drugs	Imatinib
	Gefitinib
	Erlotinib

(from Fujita, K. Cytochrome P450 and anticancer drugs. *Curr Drug Metab.* 2006 Jan;7(1):23-37.)

1.3 Regulation of HSCs fate by the BM microenvironment's control of RA

Early observations during the nineteenth century recognized that anemia often occurred in individuals with night blindness, unknowingly establishing the first association between retinoic acid (RA) and hematopoiesis [76]. Definitive evidence emerged almost 100 years ago, when it was noted that vitamin A-deficient rats displayed a reduction in hematopoietic cells in the BM [77]. Subsequent clinical studies demonstrated that vitamin A supplementation improved anemia and reduced susceptibility to infections in children. More recently, studies showing the ability of RA to differentiate promyelocytes [78, 79], together with the dramatic clinical response to ATRA of patients with APL [80-82], suggested a role for RA in myeloid differentiation. Despite these established associations, the exact function of the RA pathway in HSC regulation remained unclear, and studies have shown contradictory results, with data showing that both, inhibition or stimulation of the RA maintains HSC [83-85].

Previously, our group compared genome-wide expression data between human HSCs (CD34+ CD38−) and hematopoietic progenitor cells (HPCs) (CD34+ CD38+), to investigate molecular pathways associated with HSC self-renewal and differentiation. [86]. This analysis demonstrated that the RA pathway is differentially regulated in these two compartments. Although HSCs highly express the upstream components of the RA pathway, including RARα and ALDH1A1, the downstream targets were absent. On the other hand, HPCs show an active RA downstream pathway, with strong expression of its target genes. Therefore, despite being able to respond to RA, quiescent HSCs appear to lack the appropriate signal; when the signal is provided, the pathway is promptly

activated and HSCs differentiate to become HPCs. These data suggest that HSCs are intrinsically programmed to respond to RA and differentiate, but somehow are prevented from doing so within the BM niche.

A role for the microenvironment's regulation of RA in stem-cell fate has been described in a different system: the embryonic gonad [87]. Whether germ cells develop as oocytes or spermatogonia depends on the time at which they enter meiosis. In both sexes, germ cells are programmed to enter meiosis and become oocytes during the embryonic period in response to RA produced by the mesonephroi. However, in the embryonic testis, RA is inactivated by CYP26B1 expressed in Sertoli cells, preventing meiosis entry until after birth, at which time germ cells become spermatogonia. Thus, germ-cells are intrinsically programmed to become oocytes, unless prevented from doing so by the microenvironment's regulation of RA.

Similar to its role in germ cell biology, our group demonstrated that the microenvironment's regulation of RA is essential to determine the fate of HSCs. Consistent with this, when HSCs are removed from their niche, they rapidly differentiate, acquire a HPC phenotype (CD34+ CD38+) and loss their ability to self-renew [86, 88]. However, inhibition of RA signaling allows not only HSC maintenance but even expansion, as determined by the number of phenotypic CD34+ CD38- in culture, their progenitor output (CFU-Cs) and their ability to form cobblestones after 8 weeks of co-culture with stroma cells. Most importantly, suppression of RA signaling expands functional HSC able to self-renew and differentiate, as evidenced by increased

engraftment of primary and secondary recipients.

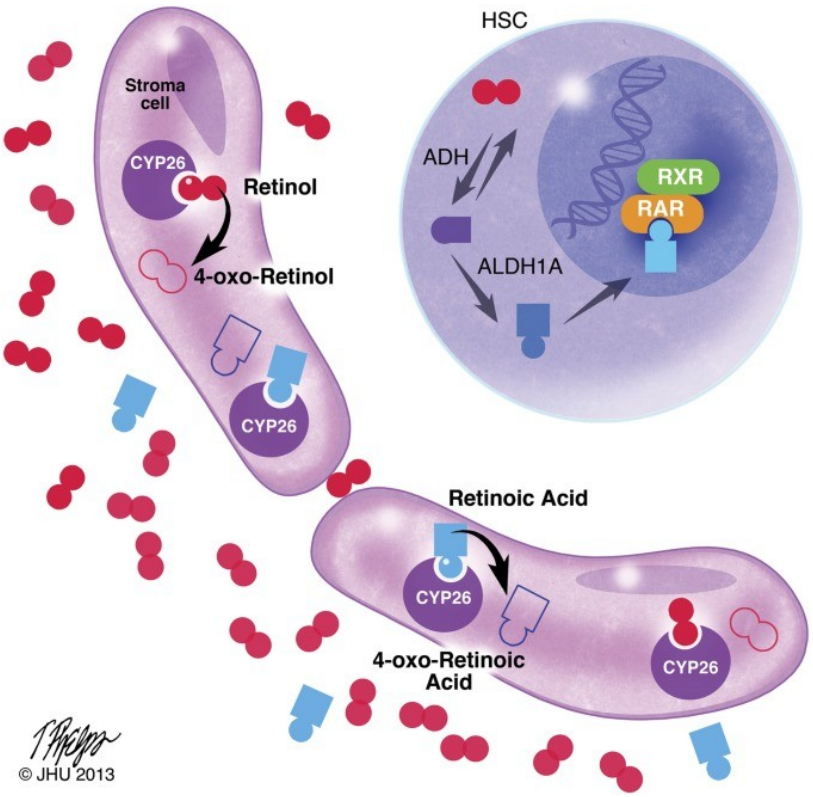

Figure 1.5. Working model of retinoid signaling in the stem-cell niche. Retinoids are present in plasma at nanomolar (RA) to micromolar (vitamin A) concentrations (28). CYP26 enzymes present in the bone marrow stroma oxidize both RA and vitamin A to their inactive 4-oxo-congeners. Accordingly, the stem-cell niche represents a retinoid-low "sanctuary" that promotes HSC quiescence, maintenance, and self-renewal. (from Ghiaur, G et al. Regulation of human hematopoietic stem cell self-renewal by the microenvironment's control of retinoic acid signaling. *Proc Natl Acad Sci USA*. 2013 Oct 1;110(40):16121-6.)

It has been known for long that bone marrow stroma cells have the ability to maintain HSCs in culture [88, 89]. Our group discovered that analogous to Sertoli cells, the BM niche regulates HSCs fate by control of RA signaling [86]. In the presence of BM MSCs, HSCs maintain their self-renewal and multilineage reconstitution capacity for several weeks. However, when CYP26 in blocked, and BM MSCs loss their ability to inactivate RA, HSCs rapidly differentiate. This high sensitivity of HSCs to local changes in RA concentration can be explained by their constitutive expression of the upstream RA pathway components, including ALDH1A and RARα. Consequently, small changes in local RA induce a rapid response in HSCs. This study suggests that local control of RA levels by the BM niche is essential to the maintenance of HSCs. More importantly, it provides the basis for manipulating the RA pathway as a potential tool for clinical HSCs expansion.

1.4 Significances of this thesis study

As outlined above, CYP enzymes have been implicated in drug metabolism and HSCs maintenance in the BM. It is particularly interesting to study the role of CYP26 and CYP3A4 plays in the BM microenvironment of hematological disorders, when there is presence of therapeutic drugs. In Chapter 2, we showed the expression of CYP26 in the MSCs in human BM confers AML cells cell-extrinsic resistance to RA treatment, protecting AML cells from differentiation. This resistance was reversed when CYP26 inhibitor was applied to the MSCs. These results drew attention to CYP26 in the MSCs as a possible therapeutic target to improve response of AML patients to RA. In Chapter 3, we investigated the changes of CYP3A4 and some other chemotherapy drug metabolizing enzyme along with chemotherapy, and found the expression level of CYP3A4 and protection to AML cells of MSCs is induced by the commonly used chemotherapy drugs in AML patients. Further, this protection effect is blocked by CYP3A4 inhibitor. Our results suggest that the chemotherapy drugs given to AML patients make the BM microenvironment a more drug resistant one. It suggests potential therapeutic windows for CYP3A4 targeting agents in clinical studies testing these agents in combination with chemotherapy.

1.5 References

1. Till, J.E. and C.E. Mc, *A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.* Radiat Res, 1961. **14**: p. 213-22.
2. Weissman, I.L., *Stem cells: units of development, units of regeneration, and units in evolution.* Cell, 2000. **100**(1): p. 157-68.
3. Mendelson, A. and P.S. Frenette, *Hematopoietic stem cell niche maintenance during homeostasis and regeneration.* Nat Med, 2014. **20**(8): p. 833-46.
4. Morrison, S.J. and D.T. Scadden, *The bone marrow niche for haematopoietic stem cells.* Nature, 2014. **505**(7483): p. 327-34.
5. Lane, S.W., D.T. Scadden, and D.G. Gilliland, *The leukemic stem cell niche: current concepts and therapeutic opportunities.* Blood, 2009. **114**(6): p. 1150-7.
6. Schepers, K., T.B. Campbell, and E. Passegue, *Normal and leukemic stem cell niches: insights and therapeutic opportunities.* Cell Stem Cell, 2015. **16**(3): p. 254-67.
7. Ghiaur, G., J. Gerber, and R.J. Jones, *Concise review: Cancer stem cells and minimal residual disease.* Stem Cells, 2012. **30**(1): p. 89-93.
8. Lapidot, T., et al., *A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.* Nature, 1994. **367**(6464): p. 645-8.
9. Wang, J.C. and J.E. Dick, *Cancer stem cells: lessons from leukemia.* Trends Cell Biol, 2005. **15**(9): p. 494-501.
10. Dean, M., T. Fojo, and S. Bates, *Tumour stem cells and drug resistance.* Nat Rev Cancer, 2005. **5**(4): p. 275-84.
11. Ghiaur, G., M. Wroblewski, and S. Loges, *Acute Myelogenous Leukemia and its*

Microenvironment: A Molecular Conversation. Semin Hematol, 2015. **52**(3): p. 200-6.

12. Krause, D.S., D.T. Scadden, and F.I. Preffer, *The hematopoietic stem cell niche--home for friend and foe?* Cytometry B Clin Cytom, 2013. **84**(1): p. 7-20.
13. Schofield, R., *The relationship between the spleen colony-forming cell and the haemopoietic stem cell.* Blood Cells, 1978. **4**(1-2): p. 7-25.
14. Bonnet, D. and J.E. Dick, *Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell.* Nat Med, 1997. **3**(7): p. 730-7.
15. Haase, D., et al., *Evidence for malignant transformation in acute myeloid leukemia at the level of early hematopoietic stem cells by cytogenetic analysis of CD34+ subpopulations.* Blood, 1995. **86**(8): p. 2906-12.
16. Eppert, K., et al., *Stem cell gene expression programs influence clinical outcome in human leukemia.* Nat Med, 2011. **17**(9): p. 1086-93.
17. Heuser, M., et al., *Modeling the functional heterogeneity of leukemia stem cells: role of STAT5 in leukemia stem cell self-renewal.* Blood, 2009. **114**(19): p. 3983-93.
18. Guezguez, B., et al., *Regional localization within the bone marrow influences the functional capacity of human HSCs.* Cell Stem Cell, 2013. **13**(2): p. 175-89.
19. Lo Celso, C., et al., *Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche.* Nature, 2009. **457**(7225): p. 92-6.
20. Mendez-Ferrer, S., et al., *Mesenchymal and haematopoietic stem cells form a unique bone marrow niche.* Nature, 2010. **466**(7308): p. 829-34.

21. Sugiyama, T., et al., *Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches.* Immunity, 2006. **25**(6): p. 977-88.

22. Ding, L. and S.J. Morrison, *Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches.* Nature, 2013. **495**(7440): p. 231-5.

23. Zhou, B.O., et al., *Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow.* Cell Stem Cell, 2014. **15**(2): p. 154-68.

24. Kiel, M.J., et al., *SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells.* Cell, 2005. **121**(7): p. 1109-21.

25. Taichman, R.S. and S.G. Emerson, *The role of osteoblasts in the hematopoietic microenvironment.* Stem Cells, 1998. **16**(1): p. 7-15.

26. Taichman, R.S., M.J. Reilly, and S.G. Emerson, *Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures.* Blood, 1996. **87**(2): p. 518-24.

27. Calvi, L.M., et al., *Osteoblastic cells regulate the haematopoietic stem cell niche.* Nature, 2003. **425**(6960): p. 841-6.

28. Visnjic, D., et al., *Hematopoiesis is severely altered in mice with an induced osteoblast deficiency.* Blood, 2004. **103**(9): p. 3258-64.

29. Zhang, J., et al., *Identification of the haematopoietic stem cell niche and control of the niche size.* Nature, 2003. **425**(6960): p. 836-41.

30. Bruns, I., et al., *Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion.* Nat Med, 2014. **20**(11): p. 1315-20.

31. Zhao, M., et al., *Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells.* Nat Med, 2014. **20**(11): p. 1321-6.

32. Chow, A., et al., *CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress.* Nat Med, 2013. **19**(4): p. 429-36.

33. Burger, J.A. and A. Peled, *CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers.* Leukemia, 2009. **23**(1): p. 43-52.

34. Meads, M.B., L.A. Hazlehurst, and W.S. Dalton, *The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance.* Clin Cancer Res, 2008. **14**(9): p. 2519-26.

35. Ishikawa, F., et al., *Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region.* Nat Biotechnol, 2007. **25**(11): p. 1315-21.

36. Saito, Y., et al., *Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML.* Nat Biotechnol, 2010. **28**(3): p. 275-80.

37. Sipkins, D.A., et al., *In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment.* Nature, 2005. **435**(7044): p. 969-73.

38. Spiegel, A., et al., *Unique SDF-1-induced activation of human precursor-B ALL cells as a result of altered CXCR4 expression and signaling.* Blood, 2004. **103**(8): p. 2900-7.

39. Rombouts, E.J., et al., *Relation between CXCR-4 expression, Flt3 mutations, and*

unfavorable prognosis of adult acute myeloid leukemia. Blood, 2004. **104**(2): p. 550-7.

40. Matsunaga, T., et al., *Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia.* Nat Med, 2003. **9**(9): p. 1158-65.

41. Shalapour, S., et al., *High VLA-4 expression is associated with adverse outcome and distinct gene expression changes in childhood B-cell precursor acute lymphoblastic leukemia at first relapse.* Haematologica, 2011. **96**(11): p. 1627-35.

42. Azab, A.K., et al., *CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy.* Blood, 2009. **113**(18): p. 4341-51.

43. Burger, J.A. and T.J. Kipps, *CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment.* Blood, 2006. **107**(5): p. 1761-7.

44. Burger, J.A., M. Burger, and T.J. Kipps, *Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells.* Blood, 1999. **94**(11): p. 3658-67.

45. Nervi, B., et al., *Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100.* Blood, 2009. **113**(24): p. 6206-14.

46. Passaro, D., et al., *CXCR4 Is Required for Leukemia-Initiating Cell Activity in T Cell Acute Lymphoblastic Leukemia.* Cancer Cell, 2015. **27**(6): p. 769-79.

47. Bjorklund, C.C., et al., *Evidence of a role for CD44 and cell adhesion in mediating resistance to lenalidomide in multiple myeloma: therapeutic*

implications. Leukemia, 2014. **28**(2): p. 373-83.

48. Jin, L., et al., *Targeting of CD44 eradicates human acute myeloid leukemic stem cells.* Nat Med, 2006. **12**(10): p. 1167-74.

49. Krause, D.S., et al., *Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells.* Nat Med, 2006. **12**(10): p. 1175-80.

50. Guengerich, F.P., *Cytochrome p450 and chemical toxicology.* Chem Res Toxicol, 2008. **21**(1): p. 70-83.

51. Lamba, J.K., et al., *Common allelic variants of cytochrome P4503A4 and their prevalence in different populations.* Pharmacogenetics, 2002. **12**(2): p. 121-32.

52. Murray, G.I., *The role of cytochrome P450 in tumour development and progression and its potential in therapy.* J Pathol, 2000. **192**(4): p. 419-26.

53. Patterson, L.H. and G.I. Murray, *Tumour cytochrome P450 and drug activation.* Curr Pharm Des, 2002. **8**(15): p. 1335-47.

54. Relling, M.V., et al., *Human cytochrome P450 metabolism of teniposide and etoposide.* J Pharmacol Exp Ther, 1992. **261**(2): p. 491-6.

55. Marre, F., et al., *Hepatic biotransformation of docetaxel (Taxotere) in vitro: involvement of the CYP3A subfamily in humans.* Cancer Res, 1996. **56**(6): p. 1296-302.

56. Sonnichsen, D.S., et al., *Variability in human cytochrome P450 paclitaxel metabolism.* J Pharmacol Exp Ther, 1995. **275**(2): p. 566-75.

57. Mathijssen, R.H., et al., *Clinical pharmacokinetics and metabolism of irinotecan (CPT-11).* Clin Cancer Res, 2001. **7**(8): p. 2182-94.

58. Schoeff, L., *Vitamin A.* Am J Med Technol, 1983. **49**(6): p. 447-52.

59. Sklan, D., *Vitamin A in human nutrition.* Prog Food Nutr Sci, 1987. **11**(1): p. 39-55.

60. Duester, G., F.A. Mic, and A. Molotkov, *Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid.* Chem Biol Interact, 2003. **143-144**: p. 201-10.

61. Theodosiou, M., V. Laudet, and M. Schubert, *From carrot to clinic: an overview of the retinoic acid signaling pathway.* Cell Mol Life Sci, 2010. **67**(9): p. 1423-45.

62. Thatcher, J.E. and N. Isoherranen, *The role of CYP26 enzymes in retinoic acid clearance.* Expert Opin Drug Metab Toxicol, 2009. **5**(8): p. 875-86.

63. Marill, J., et al., *Identification of human cytochrome P450s involved in the formation of all-trans-retinoic acid principal metabolites.* Mol Pharmacol, 2000. **58**(6): p. 1341-8.

64. McSorley, L.C. and A.K. Daly, *Identification of human cytochrome P450 isoforms that contribute to all-trans-retinoic acid 4-hydroxylation.* Biochem Pharmacol, 2000. **60**(4): p. 517-26.

65. Nadin, L. and M. Murray, *Participation of CYP2C8 in retinoic acid 4-hydroxylation in human hepatic microsomes.* Biochem Pharmacol, 1999. **58**(7): p. 1201-8.

66. Thatcher, J.E., A. Zelter, and N. Isoherranen, *The relative importance of CYP26A1 in hepatic clearance of all-trans retinoic acid.* Biochem Pharmacol, 2010. **80**(6): p. 903-12.

67. Thatcher, J.E., et al., *Substrate specificity and ligand interactions of CYP26A1,*

the human liver retinoic acid hydroxylase. Mol Pharmacol, 2011. **80**(2): p. 228-39.

68. Topletz, A.R., et al., *Comparison of the function and expression of CYP26A1 and CYP26B1, the two retinoic acid hydroxylases.* Biochem Pharmacol, 2012. **83**(1): p. 149-63.

69. Ross, A.C. and R. Zolfaghari, *Cytochrome P450s in the regulation of cellular retinoic acid metabolism.* Annu Rev Nutr, 2011. **31**: p. 65-87.

70. Xi, J. and Z. Yang, *Expression of RALDHs (ALDH1As) and CYP26s in human tissues and during the neural differentiation of P19 embryonal carcinoma stem cell.* Gene Expr Patterns, 2008. **8**(6): p. 438-42.

71. Ahluwalia, B., K. Gambhir, and H. Sekhon, *Distribution of labeled retinyl acetate and retinoic acid in rat and human testes. A possible site of retinyl acetate incorporation in rat testes.* J Nutr, 1975. **105**(4): p. 467-74.

72. Kurlandsky, S.B., et al., *Plasma delivery of retinoic acid to tissues in the rat.* J Biol Chem, 1995. **270**(30): p. 17850-7.

73. McCormick, A.M., K.D. Kroll, and J.L. Napoli, *13-cis-retinoic acid metabolism in vivo. The major tissue metabolites in the rat have the all-trans configuration.* Biochemistry, 1983. **22**(16): p. 3933-40.

74. Fujita, K., *Cytochrome P450 and anticancer drugs.* Curr Drug Metab, 2006. **7**(1): p. 23-37.

75. Kacevska, M., et al., *Inflammation and CYP3A4-mediated drug metabolism in advanced cancer: impact and implications for chemotherapeutic drug dosing.* Expert Opin Drug Metab Toxicol, 2008. **4**(2): p. 137-49.

76. Semba, R.D. and M.W. Bloem, *The anemia of vitamin A deficiency: epidemiology*

and pathogenesis. Eur J Clin Nutr, 2002. **56**(4): p. 271-81.

77. Findlay, A.B., *Bone marrow changes in the post mortem interval.* J Forensic Sci Soc, 1976. **16**(3): p. 213-8.

78. Gratas, C., et al., *Retinoid acid supports granulocytic but not erythroid differentiation of myeloid progenitors in normal bone marrow cells.* Leukemia, 1993. **7**(8): p. 1156-62.

79. Tobler, A., M.I. Dawson, and H.P. Koeffler, *Retinoids. Structure-function relationship in normal and leukemic hematopoiesis in vitro.* J Clin Invest, 1986. **78**(1): p. 303-9.

80. Castaigne, S., et al., *All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results.* Blood, 1990. **76**(9): p. 1704-9.

81. Huang, M.E., et al., *Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia.* Blood, 1988. **72**(2): p. 567-72.

82. Warrell, R.P., Jr., et al., *Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid).* N Engl J Med, 1991. **324**(20): p. 1385-93.

83. Collins, S.J., *The role of retinoids and retinoic acid receptors in normal hematopoiesis.* Leukemia, 2002. **16**(10): p. 1896-905.

84. Purton, L.E., I.D. Bernstein, and S.J. Collins, *All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells.* Blood, 2000. **95**(2): p. 470-7.

85. Tsai, S., et al., *Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development.* Genes Dev, 1994. **8**(23): p. 2831-

41.

86. Ghiaur, G., et al., *Regulation of human hematopoietic stem cell self-renewal by the microenvironment's control of retinoic acid signaling.* Proc Natl Acad Sci U S A, 2013. **110**(40): p. 16121-6.

87. Bowles, J., et al., *Retinoid signaling determines germ cell fate in mice.* Science, 2006. **312**(5773): p. 596-600.

88. Moore, K.A., H. Ema, and I.R. Lemischka, *In vitro maintenance of highly purified, transplantable hematopoietic stem cells.* Blood, 1997. **89**(12): p. 4337-47.

89. Gao, J., et al., *Characterization of OP9 as authentic mesenchymal stem cell line.* J Genet Genomics, 2010. **37**(7): p. 475-82.

CHAPTER 2

All-Trans Retinoic Acid Activity in Acute Myeloid Leukemia: Role of Cytochrome P450 Enzyme Expression by the Microenvironment

All-Trans Retinoic Acid Activity in Acute Myeloid Leukemia: Role of Cytochrome P450 Enzyme Expression by the Microenvironment

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2.1 Abstract

Differentiation therapy with all-trans retinoic acid (atRA) has markedly improved outcome in acute promyelocytic leukemia (APL) but has had little clinical impact in other AML subtypes. Cell intrinsic mechanisms of resistance have been previously reported, yet the majority of AML blasts are sensitive to atRA in vitro. Even in APL, single agent atRA induces remission without cure. The microenvironment expression of cytochrome P450 (CYP)26, a retinoid-metabolizing enzyme was shown to determine normal hematopoietic stem cell fate. Accordingly, we hypothesized that the bone marrow (BM) microenvironment is responsible for difference between in vitro sensitivity and in vivo resistance of AML to atRA-induced differentiation. We observed that the pro-differentiation effects of atRA on APL and non-APL AML cells as well as on leukemia stem cells from clinical specimens were blocked by BM stroma. In addition, BM stroma produced a precipitous drop in atRA levels. Inhibition of CYP26 rescued atRA levels and AML cell sensitivity in the presence of stroma. Our data suggest that stromal CYP26 activity creates retinoid low sanctuaries in the BM that protect AML cells from systemic atRA therapy. Inhibition of CYP26 provides new opportunities to expand the clinical activity of atRA in both APL and non-APL AML.

2.2 Introduction

Acute myeloid leukemia (AML) is characterized by impaired differentiation and uncontrolled proliferation with subsequent accumulation of immature cells (blasts). Even though the treatment results in AML have improved over the past 30 years, more than 50% of young adults and 90% of older patients die of their disease [1]. Advances in the treatment of one AML subtype, acute promyelocytic leukemia (APL), raised hopes that all-trans retinoic acid (atRA)-based therapies might improve outcomes in other AML subtypes. In APL, the C-terminus of retinoic acid receptor α (RARα) on chromosome 17 is most often fused with N-terminus of promyelocytic leukemia protein (PML) on chromosome 15 [2]. The resultant fusion protein, PML-RARα has a dominant negative effect on retinoic acid signaling and blocks differentiation by recruiting abnormal transcription factors and histone-modifying enzymes to critical genes. atRA when used at pharmacological concentrations is able to bind PML-RARα and overcome its inhibitory effects, thus allowing transcription of target genes. APL progenitors exposed to atRA in vitro or during clinical treatment will continue their differentiation program into neutrophil which eventually senesce.

The introduction of atRA in western medicine clinical protocols in 1980's changed the face of APL from one of the most malignant types of AML to the most curable [3]. Although the PML-RARα translocation appears to enhance the sensitivity of APL to atRA and several intrinsic mechanisms of atRA resistance have been identified, including overexpression of Tal1, expression of PRAME as well as epigenetic silencing or mutation of RARα [4-7], the majority of non-APL AMLs and even other cancers remain

sensitive to atRA in vitro [4, 8-12]. Several clinical trials have even suggested a clinical benefit for atRA in at least some subtypes of AML [13-15], although most trials have not confirmed these results [16-18]. It is unclear why atRA has activity against non-APL AML in vitro, but limited clinical activity. Moreover, atRA as a single agent complete remissions (CRs) in APL patients, but all patients eventually relapse [19] with a median duration of CR of about 5 months [20]. Thus even in APL there exists minimal residual disease (MRD) that remains resistant to atRA therapy [19]. While combinations of atRA with chemotherapy or arsenic trioxide eliminates MRD in APL and produces cures [21], understanding the mechanism responsible for persistence of MRD in APL patients treated with atRA monotherapy may have important implications for expanding atRA-based therapies to non-APL AML.

RA's precursor, vitamin A (retinol), plays unique roles in mammalian ontogeny and homeostasis across multiple cellular systems [22]. Since both RA deficiency as well as excess has deleterious effects, some incompatible with life, organisms have developed feedback mechanisms to control retinoid levels. Thus, tissue levels of RA reflect the balance between biosynthesis from vitamin A and inactivation, mostly via cytochrome P450 (CYP) 26 family. While hepatic CYP26 plays an important role in maintaining systemic retinoid homeostasis [23], recent reports have also implicated these enzymes in local control of RA signaling in the microenvironment. In fetal gonads, Sertoli cell expression of CYP26B1 determines the fate of the germ cells through modulating atRA bioavailability [24]. We recently found that the bone marrow (BM) microenvironment similarly expresses CYP26, which protects human hematopoietic stem cells (HSCs) from

physiological retinoid-induced differentiation and promotes their self-renewal [25].

Here we evaluate if stromal CYP26 also protects leukemia cells from pharmacological levels of atRA. We found that BM stroma degraded pharmacological concentrations of atRA, rendering even sensitive APL cells resistant to atRA. Moreover, non-APL leukemia cells were also highly sensitive to atRA treatment in the absence of BM stroma, but became resistant in stromal co-culture conditions; inhibition of CYP26 reversed the stromal-mediated atRA resistance.

2.3 Materials and Methods

2.3.1 Cell lines

The human APL cell line NB4 [26] was cultured in RPMI 1640 (Gibco, Rockville, MD, USA) with 2 mM L-glutamine (Life Technologies), 100 μg/mL penicillin-streptomycin (Gibco), and 10% fetal calf serum (FCS) (Sigma-Aldrich). The M2 AML cells Kasumi-1 [27] were cultured in RPMI 1640 + 20%FCS and the NPM1 mutated OCI/AML3 cells were cultured in minimum essential media (α-MEM) (Corning Cellgro) with 2 mM L-glutamine, 50 μg/mL penicillinstreptomycin, and 20% FCS. The mouse stroma OP9 cells were cultured in α-MEM + 20% FCS. CD34+CD38- cells were isolated from the cell lines as previously described. Briefly, the cells were labeled with monoclonal phycoerythrin (PE)-conjugated mouse anti-human CD34 IgG1, and allophycocyanin (APC)-conjugated mouse anti-human CD38 (all antibodies purchased from BD Biosciences, San Jose, CA, USA).

2.3.2 Isolation of $CD34^{+}CD38^{-}ALDH^{int}$ leukemia stem cells

Clinical bone marrow samples were obtained from patients with newly-diagnosed t(8;21) CBF AML granting informed consent as approved by the Johns Hopkins Medical Institutes' Institutional Review Board. The Johns Hopkins Institutional Review Board has approved these studies. CD34+ cells were isolated as we have previously describe [25, 28]. Briefly, mononuclear cells will be isolated from fresh samples by Ficoll-Paque (GE Healthcare Life Sciences, Piscataway, NJ, density = 1.077) centrifugation. CD34+ cells were selected by MiltenyiBiotec (Auburn, CA, USA) microbeads (binding the class II CD34 epitope) and column per manufacturer's instructions and cryopreserved until

further use. The thawed CD34+ cells were labeled with CD34 and CD38 as described above, and then stained with Aldefluor (Aldagen, Durham, NC) per manufacturer's guidelines. The CD34+CD38-ALDHint cells were then isolated using a FACSAria (BD Biosciences), and cultured in RPMI1640 supplemented with 10% FBS (Sigma-Aldrich), 100 μg/mL penicillin-streptomycin (P/S; Sigma), and growth factors [thrombopoietin 20 ng/mL, Stem Cell Factor 100 ng/mL, and Flt3 ligand 100 ng/mL (TSF) (all growth factors and cytokines are from Amgen)], and incubated at 37°C.

2.3.3 Isolation of primary bone marrow stroma

Primary bone marrow stroma cells were derived from normal bone marrow donors granting informed consent as approved by the Johns Hopkins Medical Institutes' Institutional Review Board, as we have previously described [25]. Briefly, mononuclear cells isolated from bone marrow of normal volunteers were cultured in FBMD1 media [IMDM media (Gibco) supplemented with 15% FBS (Sigma-Aldrich), 5% Horse serum (Sigma-Aldrich), 100 μg/mL penicillin-streptomycin (Gibco), and 10^{-4}M β mercaptoethanol (Sigma-Aldrich)] [29] at 33°C in 5%CO_2 overnight. The next day, media and cells in suspension were removed and the attached cells were washed twice with phosphate-buffered saline (PBS) (Gibco), fresh FBMD1 media was added to the flask and they were placed back 33°C in 5%CO_2. Half of the media was replaced weekly until an adherent monolayer has formed. At that time, the cells were dissociated using Trypsin (Gibco) and they were either used for further experiments or cryopreserved. The passage number of the cells was recorded with original cells labeled as P1. Experiments presented in this paper were performed using bone marrow stroma at passages 2–4.

2.3.4 Co-culture system

For co-culture conditions, 24-well plates (Sigma) were coated with 0.1% Gelatin (Sigma) in PBS for 30 min. at 37°C. Gelatin solution was removed and stroma cells were seeded at a density of $5x10^4$ cells/well and cultured until a confluent monolayer was obtained. Subsequently, $2.5x10^4$ NB4, and OCI/AML3 cells and 5x104 Kasumi-1 cells and primary patient LSCs were plated per well. The cultures were treated with or without 10^{-7}MRA (for NB4 cells) or 10^{-6}M RA (for all other AML cells) as well as 10^{-6}MR115866 (CYP26 inhibitor—generously supplied by Johnson & Johnson, R&D) for 72h.

2.3.5 Colony forming unit (CFU-C)

Clonogenic growth of AML cell lines was evaluated as we previously described [30, 31]. Briefly, previously treated cells were removed from the plate and washed with PBS to remove the respective drug. Cells were then counted using Trypan blue and plated 1 mL 1.2% methylcellulose (Sigma-Aldrich), 30% bovine serum albumin (BSA) (Sigma-Aldrich), 10^{-4} M β-mercaptoethanol (Sigma-Aldrich), and 2 mM L-glutamine (Gibco). Samples were plated in triplicate onto 35-mm^2 tissue culture dishes and incubated in a humidified atmosphere at 37°C and 5% CO_2. Colonies consisting of more than 40 cells were scored at 5–10 days using an inverted microscope.

2.3.6 Flow cytometry

The cell lines and clinical AML samples were analyzed for expression of cell surface antigens using FACS Calibur (BD Biosciences, CA, USA). Briefly, AML cells were

washed with PBS containing 0.2% BSA and stained with the following antibodies for 30 min at 4°C: phycoerythrin (PE)-conjugated mouse anti-human CD11b IgG1, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD15 IgM antibodies, FITC-conjugated mouse anti-human CD34 IgG1, PE-conjugated mouse anti-human CD38 IgG2α, and allophycocyanin (APC)-conjugated mouse anti-human CD45 IgG2β antibodies or their respective isotype controls. All antibodies were purchased from BD Biosciences. Cells will be then washed to remove unbound antibody, and evaluated using a FACS Calibur (BD Biosciences) with a minimum acquisition of 10,000 events.

2.3.7 Retinoic acid quantification

Culture media (RPMI+10%FCS), supplemented with 10-6MatRA was incubated at 37°C, 5% CO_2 humidified tissue culture incubator in the presence or absence of OP-9 bone marrow stroma with or without 10^{-6}MR115866. Media was harvested after 0h, 2h, 8h and 24h, was spun down to eliminate any potential cellular debris and was frozen and stored at -80°C until analysis. For analysis, media was extracted with two step liquid-liquid extraction as previously described [32]. RA isomers were quantified using LC-MS/MS on a AB Sciex 5500 QTRAP in MRM mode using APCI in positive ion mode as previously described [32, 33].

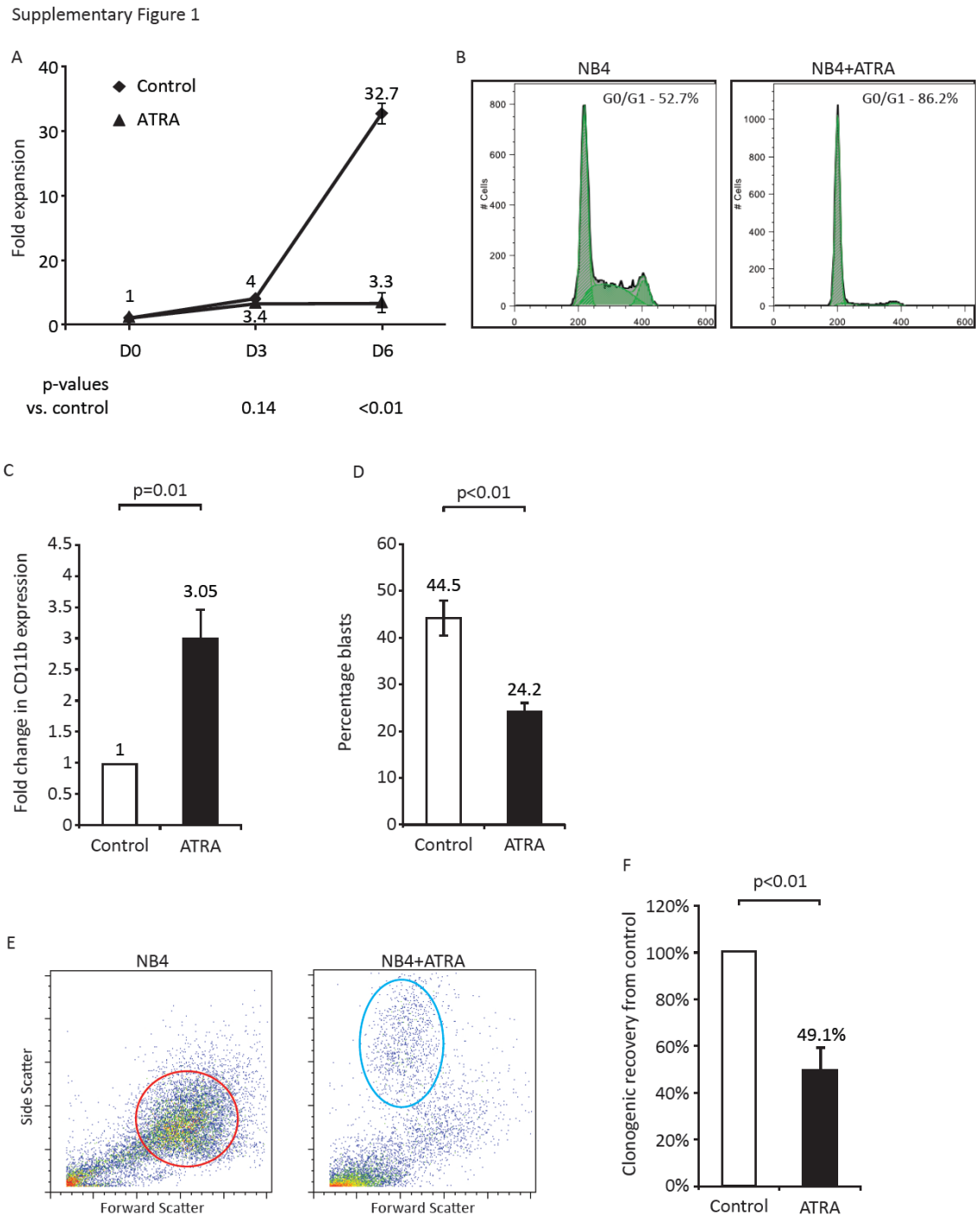
2.3.8 Statistical analysis

Statistical analysis was performed by using two-tail unpaired student t test to compare the averages of two groups and calculate the p value.

2.4 Results

2.4.1 Stromal CYP26 prevents atRA-induced differentiation of APL cells

It is well documented that atRA differentiates NB4 APL cells with subsequent decreased cellular expansion (S1A Fig), cell cycle arrest (S1B Fig), up-regulation of differentiation markers (S1C Fig), decreased blasts (S1D and S1E Fig) and clonogenic loss (S1F Fig). We recently showed that BM mesenchymal stroma protects normal HSCs from atRA-mediated differentiation through the expression of CYP26, the major mechanism of retinoid inactivation [25]. NB4 co-cultures with the mouse BM stromal line OP9 similarly blocked atRA (10^{-7}MatRA for 72h) mediated induction of CD11b expression and clonogenic loss (Fig 1A and 1B). Inhibition of CYP26 activity via R115866 restored atRA-induced up regulation of CD11b and inhibition of clonogenic activity. The addition of the CYP26 inhibitor in the absence of stroma had no effect on atRA-induced differentiation (data not shown). Primary human BM stroma also protected against atRA-induced differentiation of NB4 cells. Low passage (<P3) human primary BM stromal cultures from four normal volunteers were co-cultured with NB4 APL cells and atRA. As with the OP9 stromal cells, primary stroma blocked atRA-induced differentiation that was rescued by CYP26 inhibition (Fig 1C and 1D). Consistent with the lack of direct cytotoxic effects of ATRA on APL cells, exposure of these cells to retinoids for 72h in all the conditions analyzed resulted in no significant differences in cellular numbers.



Supplementary Fig 2.1. Effects of ATRA on NB4 APL cells. A) Cellular expansion of NB4 APL cells in the presence of 10^{-7}M ATRA. ATRA treated cells (triangles) expand 3.4±0.5 fold by Day (D)3 of cultures compared to 4±0.3 fold in control culture (p = 0.14). Continued exposure to ATRA for 6 days results in 3.3±1.6 fold expansion from D0

compared to 32.7±1.3 in control cultures ($p<0.01$). Data represent mean ± SEM of three independent experiments. B) Cell cycle analysis of NB4 APL cells treated with ATRA for 6 days. One representative experiment from three with similar results show increased cells in G0/G1 phases of cell cycle upon treatment with ATRA. C) Expression CD11b, a differentiation marker of NB4 APL cells. Upon culture in the presence of ATRA for 72h the mean fluorescence intensity (MFI) of CD11b expression of NB4 cells was 3.05±0.48 fold higher compared to control cultures. Data represent mean ±SEM of three independent experiments, $p = 0.01$. D) Effects of prolonged ATRA treatment on morphologically defined NB4 APL blasts. ATRA treated cultures have 24.2%±3.8% blasts at D6 compared to 44.5%±7.1% in control cultures. Data represent mean ± STD of four independent experiments, $p<0.01$.E) Effects of ATRA treatment for 6 days on cell size (indicated by Forward Scatter) and cytoplasmic complexity (indicated by right angle Side Scatter). Compared to control (red circle), ATRA treated cultures contain a population of cells (blue oval) that are relatively smaller and have increased cytoplasmic complexity. One representative experiment from three with similar results. F) Effects of 10^{-7}M ATRA on the clonogenic activity of NB4 APL cells. Treatment with ATRA for 72h results in 49.1%±8.9% clonogenic recovery from control cultures. Data represent mean±SEM of eight independent experiments, $p<0.01$.

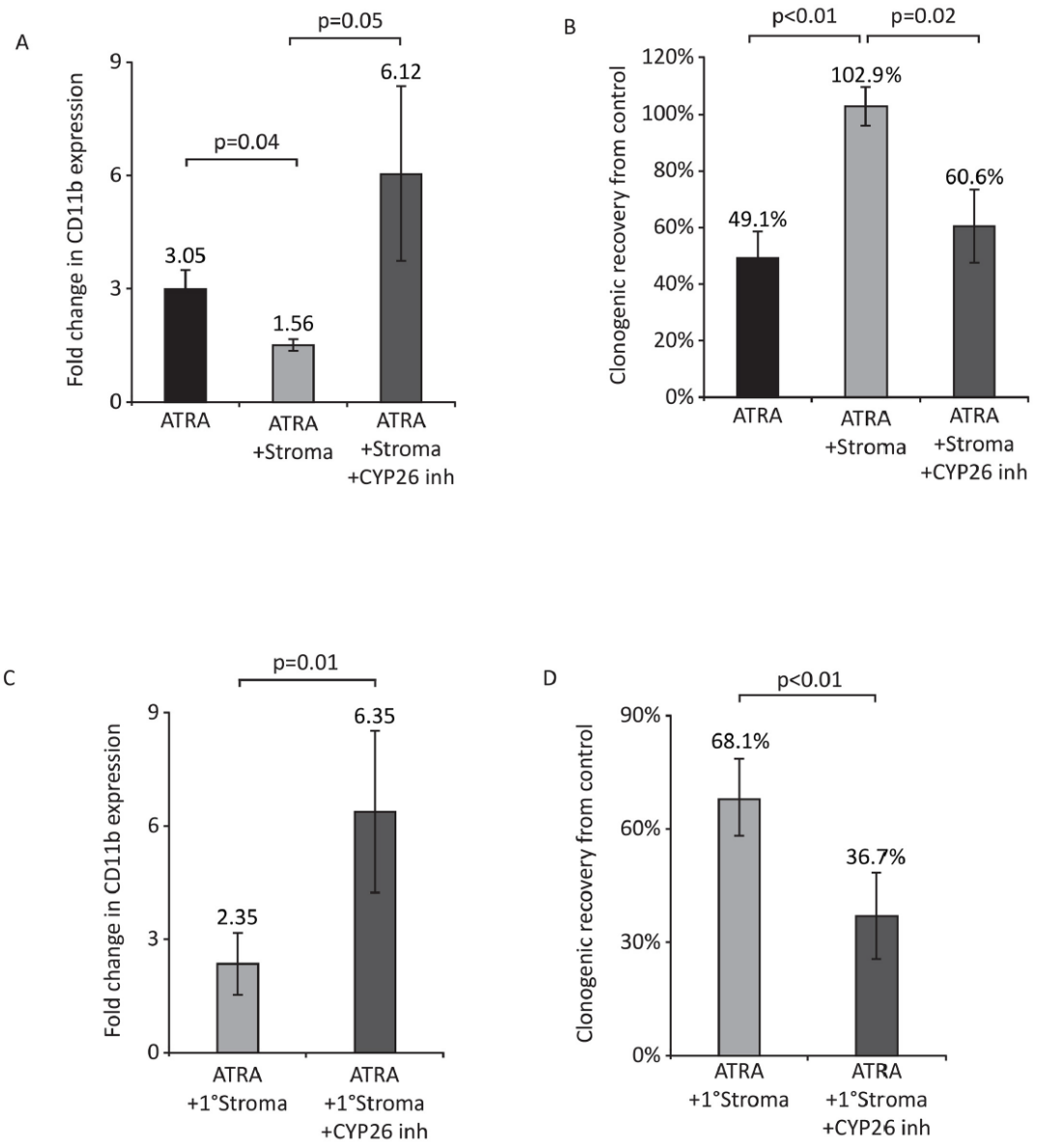


Fig 2.1. Effects of BM stroma on atRA-induced differentiation of APL cells. Effects of 10-7M ATRA±10-6M R115866 for 72h on the (A) expression of CD11b and (B) clonogenic growth of NB4 APL cells in the presence of OP9 BM stroma. Data represent mean ± SEM of three independent experiments. Effects of 10-7M atRA ± 10-6M R115866 for 72 hours on the (C) expression of CD11b and (D) clonogenic growth of NB4 APL cells in the presence of primary human BM stroma from normal donors. ATRA only controls for these conditions are presented in panels A and B respectively.

Results represent mean ± SEM of independent experiments using four individual primary stromas. Treatment of APL cells with CYP26 inhibitor with or without ATRA in the absence of stroma or in the presence of stroma without ATRA had no effect on differentiation status of these cells.

2.4.2 Stromal CYP26 metabolizes atRA

To prove that stromal CYP26 can metabolize pharmacological levels of atRA, BM stroma was incubated with 10^{-6}MatRA, and atRA was quantified in the conditioned medium by high pressure liquid chromatography—tandem mass spectrometry (HPLC-MS/MS) (Fig 2A and 2B). In the presence of BM stroma, there is a time dependent decrease in atRA levels such that at 24h only about 10% of the atRA remained ($p<0.01$). Inhibition of CYP26 by R115866 blocked the metabolism of atRA such that atRA levels were comparable to no stroma controls ($p = 0.37$) (Fig 2A). Consistent with previous reports [34], the elimination half-life of atRA in the absence of stroma was 21.5±1.9h; the presence of BM stroma decreased the half-life to only 7.6±0.4h ($p<0.01$). CYP26 inhibitor rescued atRA half-life to levels comparable to no stroma control (24.8±3.2h, $p = 0.34$ vs. control).

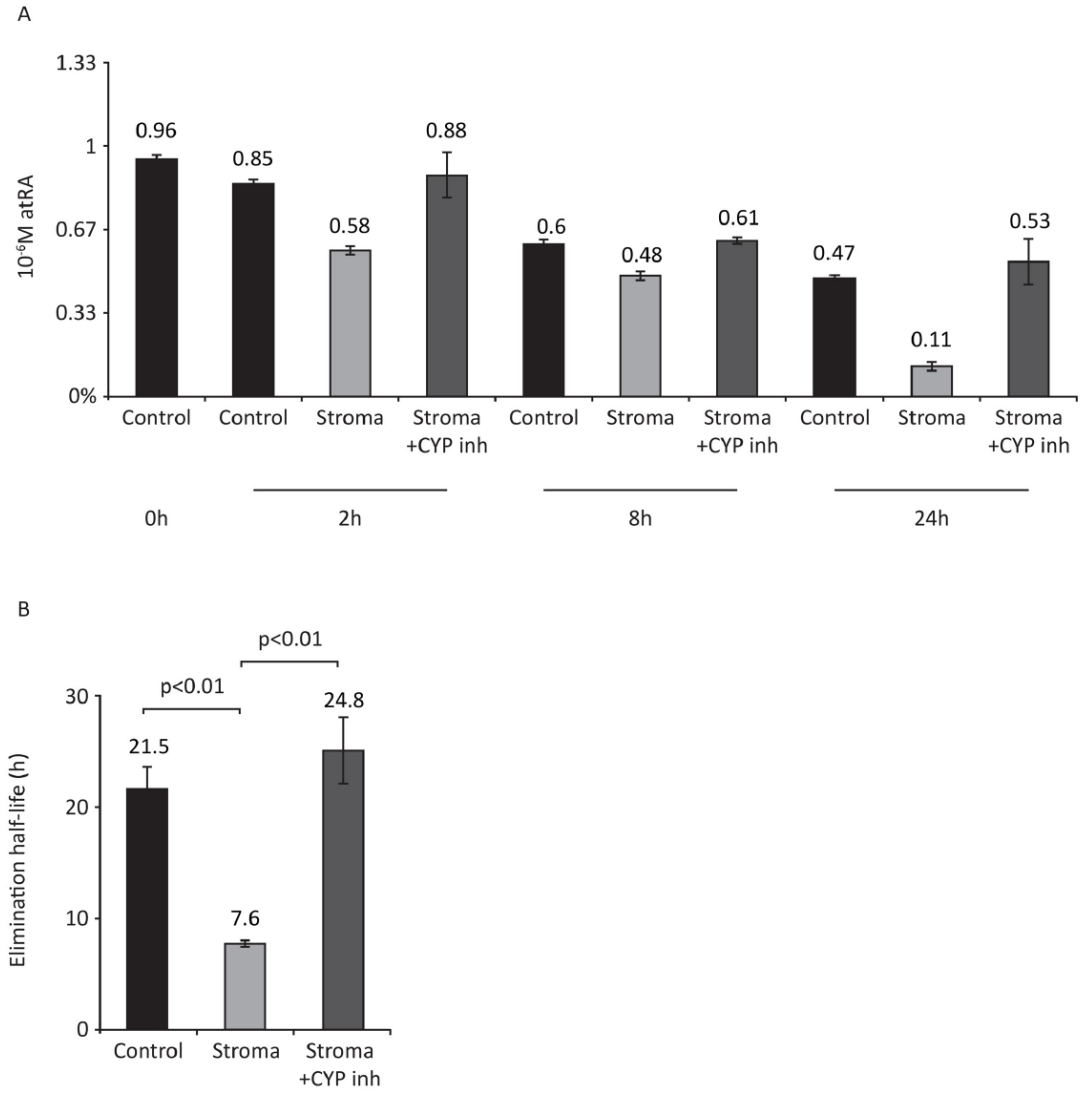


Fig 2.2. Effect of BM stroma on atRA concentrations. Media (RPMI+10%FCS) was supplemented with 10^{-6}M atRA and incubated at 37°C in a humidified incubator in 5%CO2 either in the absence of stroma (Control) or in the presence of stroma with or without 10-6M CYP26 inhibitor (R115866). A) The concentration of atRA was determined at 0h, 2h, 8h and 24h. B) Elimination half-life of atRA (t1/2 = 0.693/slope) calculated from the natural logarithms of percent atRA remaining over time. The half-life of atRA during culture conditions was 21.5±1.9h in the absence of stroma (Control), 7.6±0.4h in the presence of stroma and 24.8±3.2h in stroma+CYP inhibitor conditions.

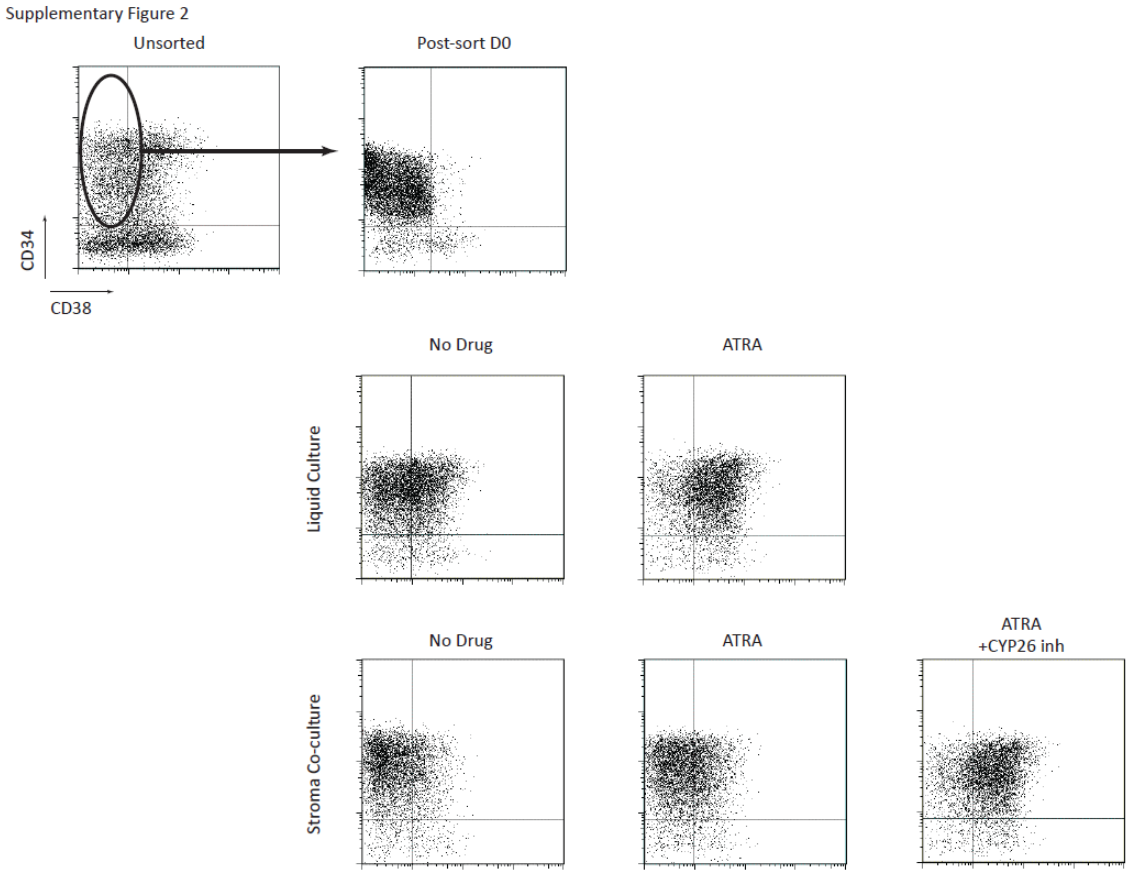
Data represent mean ± STD of three independent experiments.

2.4.3 BM stroma blocks atRA-induced differentiation of non-APL AML via CYP26

atRA has also been shown to induce differentiation of the t(8;21) core binding factor (CBF) AML cell line Kasumi-1[35], although it has shown no clinical activity in CBF AML[15–18]. About 20–30% of Kasumi-1 cells exhibit a HSC phenotype (CD34+CD38-) (S2 Fig), and culture in the presence of atRA resulted in rapid loss of CD34+CD38- compartment both phenotypically and by clonogenic recovery (Fig 3A and 3B, $p<0.01$). Kasumi-1 CD34+CD38- cells and their clonogenic activity were protected from atRA-induced differentiation when co-cultured with BM stroma (Fig 3A and 3B). This protection was rescued by inhibition of CYP26. Again, CYP26 inhibition had no effect in stromal-free cultures (data not shown).

While atRA has shown in vitro activity against NPM1-mutated AMLs [35], most studies [36, 37] have not confirmed the initial report suggesting clinical activity in this AML subtype [13]. Thus, we investigated whether the microenvironment may also play a role in these divergent findings regarding the effect of atRA in NPM1-mutated AML. NPM1 mutated AML cell line OCI-AML3, was treated with atRA in the absence or presence of BM stroma (Fig 3D). Treatment of OCI-AML3 cells with 1μMatRA for 72h resulted in 90% loss of clonogenic growth ($p<0.01$), while similar treatment in the presence of BM stroma had no effect on OCI-AML3 clonogenic recovery. Inhibition of CYP26 by R115866 overcame the protective effect of stroma against OCI-AML3 treated with atRA ($p<0.01$). Consistent with the lack of direct cytotoxic effects of ATRA on AML cells, exposure of these cells to retinoids for 72h in all the conditions analyzed resulted in no

significant differences in cellular numbers.



Supplementary Fig 2.2. Flow cytometry analysis of Kasumi-1 cells. Expression of CD34 and CD38 prior to sort (upper left plot) and post sort but prior to further culture (upper right panel). Middle and lower panels show CD34+CD38- sorted Kasumi-1 cells after 72h of culture in the absence (Liquid culture) or presence (Stroma co-culture) of BM stroma, respectively. Cells treated with 1μM atRA are presented in middle right panel and lower mid panel, and cells treated with 1μM atRA+1μMCYP26 inhibitor, R115866 are presented in lower right panel. Dead cells were gated out based on 7AAD positivity and non-hematopoietic cells (i.e. BM stroma) were dismissed based on lack of CD45 expression. This is one representative experiment from four with similar results.

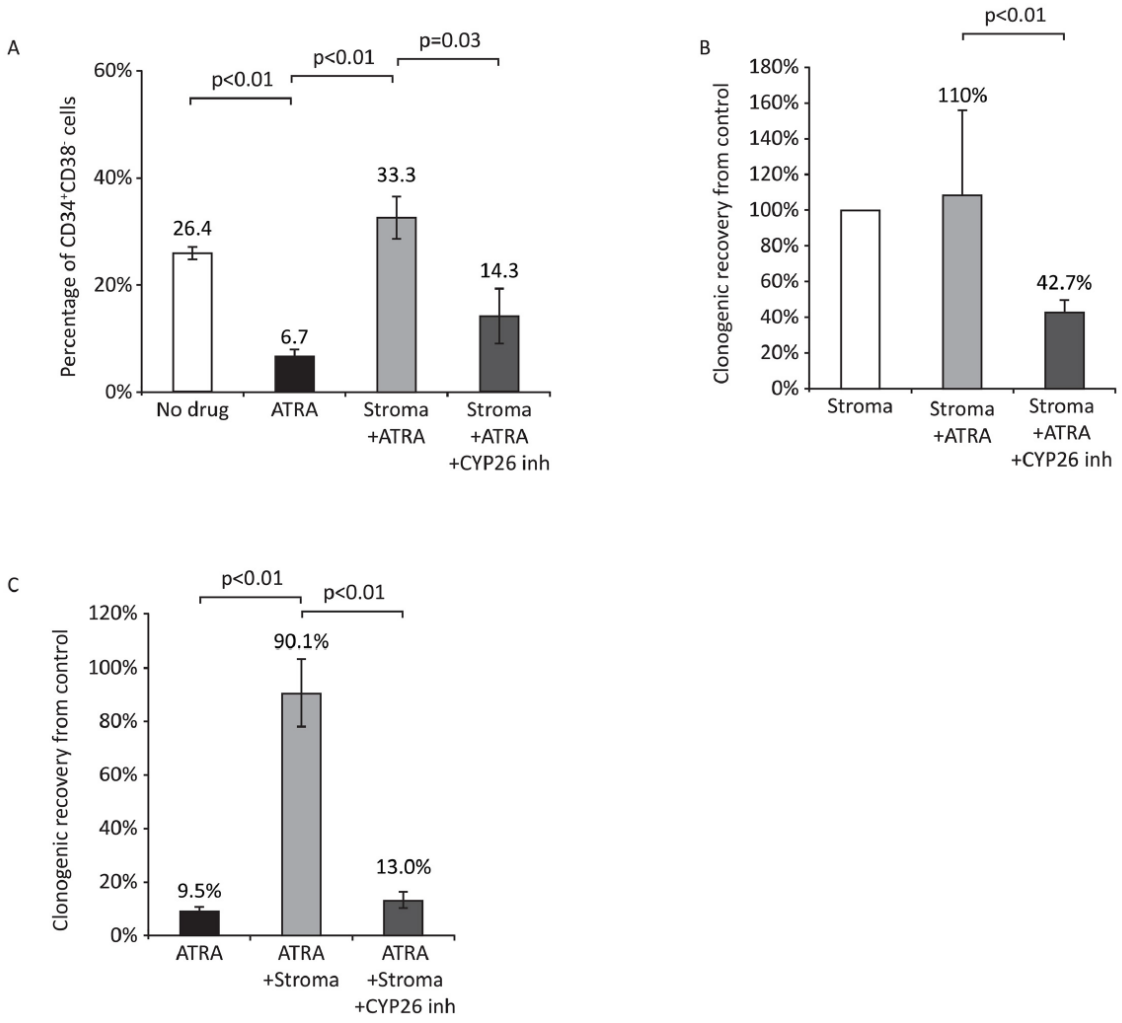


Fig 2.3. Effects of bone marrow stroma on atRA-induced differentiation of non-APL AML cells. Effects of 10^{-6}M atRA ± 10-6M R115866 for 72 hours on (A) the phenotypic differentiation and (B) clonogenic growth of Kasumi-1 cells in the presence of OP9 bone marrow stroma. CD34+CD38- Kasumi-1 cells were isolated by flow cytometry and cultured as described. Data are presented as mean ± SEM of four (A) or three (B) independent experiments respectively. C) Effects of 10^{-6}M ATRA ± 10-6M R115866 for 72 hours atRA on the clonogenic growth of OCI/AML-3 cells in the presence of OP9 bone marrow stroma. Data represent the mean ± SEM of three independent experiments. Treatment of AML cells with CYP26 inhibitor with or without ATRA in the absence of

stroma or in the presence of stroma without ATRA had no effect on differentiation status of these cells.

2.4.4 Primary leukemia stem cells (LSCs) are protected from atRA via niche CYP26

Laboratory data suggest that AML maintains the basic hierarchical structure of normal hematopoiesis; i.e., rare cells possessing self-renewal capacity, so-called LSCs, give rise to partially differentiated progeny that compose the tumor bulk but possess only limited proliferative potential [38]. Although the clinical significance of LSCs has been questioned, recent data strongly implicate LSCs (CD34+CD38- intermediate ALDH activity or ALDHint) in disease relapse; MRD was enriched for LSCs, and their presence after therapy highly correlated with subsequent clinical relapse [28]. To test if BM microenvironment protects primary LSCs from atRA, we isolated CD34+CD38-ALDH^{int} cells from the BM of patients with newly-diagnosed CBF [t(8;21)] AML. Prior to culture, these cells expressed no differentiation markers such as CD15 (Fig 4A), CD33, and CD11b (data not shown). Culture of these cells in media containing 10% serum (and about 1nM atRA) [39] led to acquisition of differentiation markers including CD15 (Fig 4A—upper middle panel). The addition of 1μMatRA induced further up regulation of CD15 (Fig 4A; upper right panel, and 4B). Culture in the presence of BM stroma inhibited acquisition of CD15 (Fig 4A, lower middle panel, and 4B), and this was rescued by inhibition of stromal CYP26 (Fig 4A, lower right panel, and 4B, $p = 0.01$).

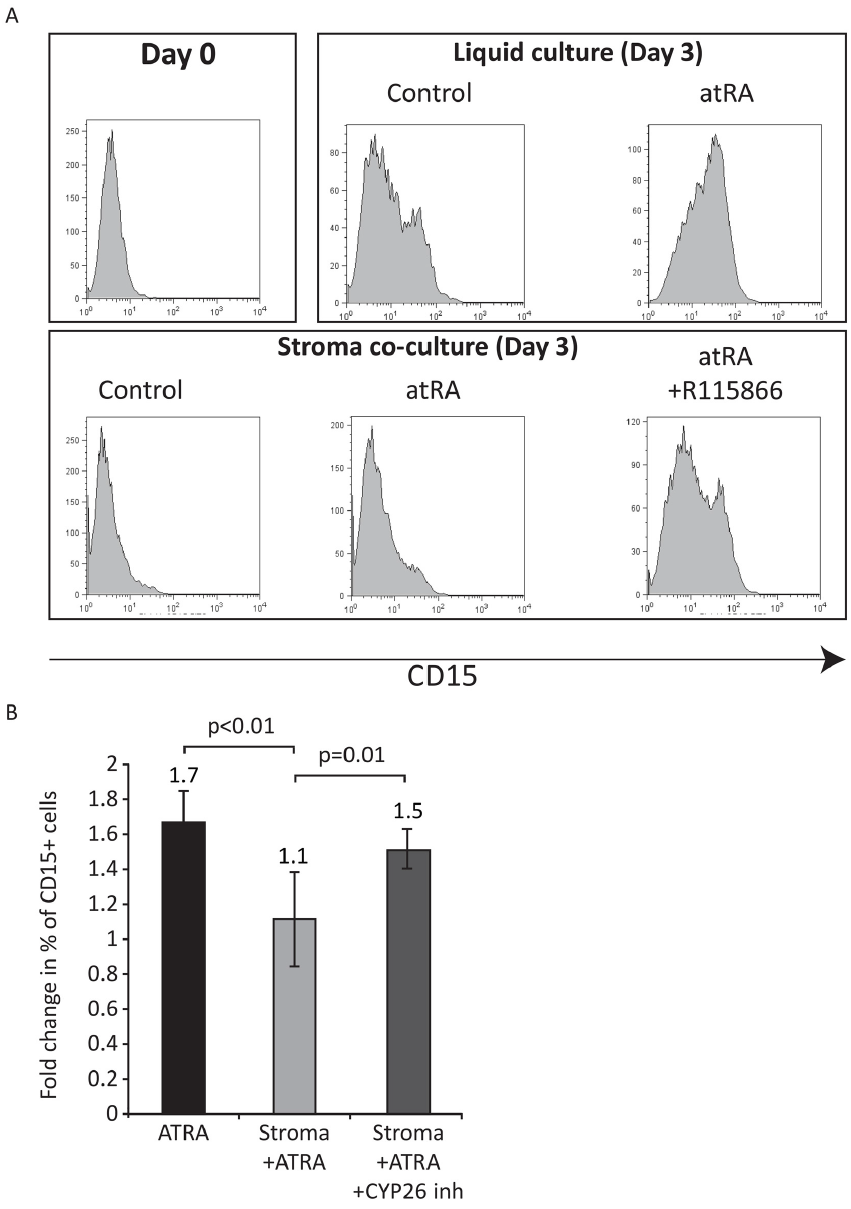


Fig 2.4. Bone marrow stromal effects on atRA-induced differentiation of primary CBF LSCs. (A) One representative experiment (from four independent patients with similar results) of CD15 expression of CBF AML CD34+CD38-ALDHint cells prior to culture (left upper panel), post 72h of culture in the absence of BM stroma (right upper panel), or in the presence of BM stroma (lower panel). Prior to culture, few if any LSCs express CD15 (range 0.1%-3%, left upper panel). After culture for 72h in

RPMI+10%FCS and thrombopoietin, kit ligand, and Flt3 ligand, a range 8.3%-41.7% of cells expressed CD15. (B) Quantitative results from all 4 experiments showing fold change of proportion of cells expressing CD15 from control cultures. Data are presented as mean ± SEM of fold change from control using CD34+CD38-ALDHint cells from four different patients with CBF AML. Treatment of LSCs with CYP26 inhibitor with or without ATRA in the absence of stroma or in the presence of stroma without ATRA had no effect on differentiation status of these cells.

2.5 Discussion

The early success seen with the introduction of atRA in treatment protocols of APL raised hope that differentiation therapy could change the face of current treatments in leukemia; this was further bolstered by evidence of atRA's activity against most non-APL AMLs in vitro [4, 8-12]. Unfortunately, initial enthusiasm has been dampened by largely negative results from clinical trials using combination atRA + chemotherapy for induction regimens in non-APL AML [16-18]. Nevertheless, preclinical data has shown that atRA was actually able to induce terminal differentiation of many subtypes of AML in vitro [40].

Our data suggest that the leukemic microenvironment could provide a potential explanation for the lack of clinical benefit of atRA despite in vitro activity, but few if any studies have actually focused on the effects of the microenvironment on atRA activity in AML. Results presented here show that even the most sensitive AML cells, including APL, become resistant to atRA treatment in the presence of stromal CYP26 activity. We previously showed that the stem cell niche CYP26 expression also protected normal HSCs from retinoids, helping to maintaining them in a quiescent, undifferentiated state [25]. Interestingly, since CYP26 is directly up-regulated by retinoids [41] in a variety of tissues including hepatocyte, intestine, endothelial cells and even leukemia [42], treatment with pharmacological doses of atRA could induce CYP26 expression by the BM niche and produce an even more protective microenvironment for LSCs. In-depth analysis of CYP26 levels in the BM microenvironment of patients with AML and how they change during therapy are warranted for further understanding of how the protective

niche changes in patients with AML. Niche inactivation of atRA could also partially explain its ability to induce terminal differentiation of the malignant promyelocytes in APL, but the inability to eliminate LSCs.

The relative effectiveness of atRA in APL may result from increased intrinsic sensitivity of APL cells comparted to non-APL. The dose of atRA (1M) needed to inhibit non-APL cells in the absence of stroma is a log higher than the similar dose (0.1M) active in APL cells (Fig 4). Nevertheless, 1M is clinically achieved with therapeutic doses of atRA [43] suggesting there are also extrinsic reasons for the lack of effectiveness of atRA in non-APL AML. Disparities in differentiation status between APL LSCs and non-APL AML LSCs [44] may translate into occupancy of distinct niches which could contribute to the differential clinical activity seen with ATRA in APL.

Our results reveal therapeutic opportunities for improving the effectiveness of retinoids in AML by overcoming the microenvironment's ability to inactivate atRA. Several CYP26 inhibitors, including R115866 used here, have safely been in clinical trials for other indications such as acne and psoriasis [45]. Systemic inhibition of CYP26 is expected to increase plasma atRA levels with potential increased toxicity. Thus, pharmacologically adjusting atRA doses to maintain safe systemic concentrations in the presence of CYP26 inhibition, should control for hepatic inhibition of the enzyme while at the same time removing the barrier to therapeutic atRA levels in the microenvironment. The synthetic retinoid tamibarotene (AM80) has activity in atRA-resistant APL and is approved in Japan for this indication [46]. The resistance of tamibarotene to CYP26 [47] may be

responsible for its activity in atRA-resistant APL. Such approaches that circumvent CYP26 in the leukemic microenvironment could expand the effectiveness of retinoid-based therapy in both APL and non-APL AML.

2.6 References

1. Estey, E. and H. Dohner, *Acute myeloid leukaemia.* Lancet, 2006. **368**(9550): p. 1894-907.
2. Dos Santos, G.A., L. Kats, and P.P. Pandolfi, *Synergy against PML-RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia.* J Exp Med, 2013. **210**(13): p. 2793-802.
3. Wang, Z.Y. and Z. Chen, *Acute promyelocytic leukemia: from highly fatal to highly curable.* Blood, 2008. **111**(5): p. 2505-15.
4. Altucci, L. and H. Gronemeyer, *The promise of retinoids to fight against cancer.* Nat Rev Cancer, 2001. **1**(3): p. 181-93.
5. Bullinger, L., et al., *PRAME-induced inhibition of retinoic acid receptor signaling-mediated differentiation--a possible target for ATRA response in AML without t(15;17).* Clin Cancer Res, 2013. **19**(9): p. 2562-71.
6. Petrie, K., A. Zelent, and S. Waxman, *Differentiation therapy of acute myeloid leukemia: past, present and future.* Curr Opin Hematol, 2009. **16**(2): p. 84-91.
7. Rice, A.M., et al., *Analysis of the relationship between Scl transcription factor complex protein expression patterns and the effects of LiCl on ATRA-induced differentiation in blast cells from patients with acute myeloid leukemia.* Leuk Res,

2004. **28**(11): p. 1227-37.

8. Lehmann, S., et al., *Effects of retinoids on cell toxicity and apoptosis in leukemic blast cells from patients with non-M3 AML.* Leuk Res, 2000. **24**(1): p. 19-25.

9. Lehmann, S., C. Paul, and H. Torma, *The expression of cellular retinoid binding proteins in non-APL leukemic cells and its association with retinoid sensitivity.* Leuk Lymphoma, 2002. **43**(4): p. 851-8.

10. Sakamoto, K., et al., *Sensitivity of MLL-rearranged AML cells to all-trans retinoic acid is associated with the level of H3K4me2 in the RARalpha promoter region.* Blood Cancer J, 2014. **4**: p. e205.

11. Sakashita, A., et al., *9-cis-retinoic acid: effects on normal and leukemic hematopoiesis in vitro.* Blood, 1993. **81**(4): p. 1009-16.

12. Seiter, K., et al., *Clinical and laboratory evaluation of all-trans retinoic acid modulation of chemotherapy in patients with acute myelogenous leukaemia.* Br J Haematol, 2000. **108**(1): p. 40-7.

13. Schlenk, R.F., et al., *Gene mutations and response to treatment with all-trans retinoic acid in elderly patients with acute myeloid leukemia. Results from the AMLSG Trial AML HD98B.* Haematologica, 2009. **94**(1): p. 54-60.

14. Schlenk, R.F., et al., *Phase III study of all-trans retinoic acid in previously untreated patients 61 years or older with acute myeloid leukemia.* Leukemia, 2004. **18**(11): p. 1798-803.

15. Venditti, A., et al., *All-trans retinoic acid and low-dose cytosine arabinoside for the treatment of 'poor prognosis' acute myeloid leukemia.* Leukemia, 1995. **9**(7): p. 1121-5.

16. Burnett, A.K., et al., *A comparison of low-dose cytarabine and hydroxyurea with or without all-trans retinoic acid for acute myeloid leukemia and high-risk myelodysplastic syndrome in patients not considered fit for intensive treatment.* Cancer, 2007. **109**(6): p. 1114-24.

17. Milligan, D.W., et al., *Fludarabine and cytosine are less effective than standard ADE chemotherapy in high-risk acute myeloid leukemia, and addition of G-CSF and ATRA are not beneficial: results of the MRC AML-HR randomized trial.* Blood, 2006. **107**(12): p. 4614-22.

18. Estey, E.H., et al., *Randomized phase II study of fludarabine + cytosine arabinoside + idarubicin +/- all-trans retinoic acid +/- granulocyte colony-stimulating factor in poor prognosis newly diagnosed acute myeloid leukemia and myelodysplastic syndrome.* Blood, 1999. **93**(8): p. 2478-84.

19. Warrell, R.P., et al., *Differentiation Therapy of Acute Promyelocytic Leukemia with Tretinoin (All-trans-Retinoic Acid).* New England Journal of Medicine, 1991. **324**(20): p. 1385-1393.

20. Wang, Z.Y., et al., *Treatment of acute promyelocytic leukemia with all-trans retinoic acid in China.* Nouv Rev Fr Hematol, 1990. **32**(1): p. 34-6.

21. Lo-Coco, F., et al., *Retinoic acid and arsenic trioxide for acute promyelocytic leukemia.* N Engl J Med, 2013. **369**(2): p. 111-21.

22. Theodosiou, M., V. Laudet, and M. Schubert, *From carrot to clinic: an overview of the retinoic acid signaling pathway.* Cell Mol Life Sci, 2010. **67**(9): p. 1423-45.

23. Ray, W.J., et al., *CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family.* J Biol Chem, 1997. **272**(30): p. 18702-8.

24. Bowles, J., et al., *Retinoid signaling determines germ cell fate in mice.* Science, 2006. **312**(5773): p. 596-600.

25. Ghiaur, G., et al., *Regulation of human hematopoietic stem cell self-renewal by the microenvironment's control of retinoic acid signaling.* Proc Natl Acad Sci U S A, 2013.

26. Lanotte, M., et al., *NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3).* Blood, 1991. **77**(5): p. 1080-6.

27. Asou, H., et al., *Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation.* Blood, 1991. **77**(9): p. 2031-6.

28. Gerber, J.M., et al., *A clinically relevant population of leukemic CD34(+)CD38(-) cells in acute myeloid leukemia.* Blood, 2012. **119**(15): p. 3571-7.

29. Breems, D.A., et al., *Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay.* Leukemia, 1994. **8**(7): p. 1095-104.

30. Bedi, A., et al., *BCR-ABL-mediated inhibition of apoptosis with delay of G2/M transition after DNA damage: a mechanism of resistance to multiple anticancer agents.* Blood, 1995. **86**(3): p. 1148-58.

31. Matsui, W.H., et al., *The role of growth factors in the activity of pharmacological differentiation agents.* Cell Growth Differ, 2002. **13**(6): p. 275-83.

32. Kane, M.A. and J.L. Napoli, *Quantification of endogenous retinoids.* Methods Mol Biol, 2010. **652**: p. 1-54.

33. Kane, M.A., et al., *Quantitative profiling of endogenous retinoic acid in vivo and*

in vitro by tandem mass spectrometry. Anal Chem, 2008. **80**(5): p. 1702-8.

34. Williams, J.B. and J.L. Napoli, *Metabolism of retinoic acid and retinol during differentiation of F9 embryonal carcinoma cells.* Proc Natl Acad Sci U S A, 1985. **82**(14): p. 4658-62.

35. Balusu, R., et al., *Targeting levels or oligomerization of nucleophosmin 1 induces differentiation and loss of survival of human AML cells with mutant NPM1.* Blood, 2011. **118**(11): p. 3096-106.

36. Burnett, A.K., et al., *The impact on outcome of the addition of all-trans retinoic acid to intensive chemotherapy in younger patients with nonacute promyelocytic acute myeloid leukemia: overall results and results in genotypic subgroups defined by mutations in NPM1, FLT3, and CEBPA.* Blood, 2010. **115**(5): p. 948-56.

37. Nazha, A., et al., *The Addition of All-Trans Retinoic Acid to Chemotherapy May Not Improve the Outcome of Patient with NPM1 Mutated Acute Myeloid Leukemia.* Front Oncol, 2013. **3**: p. 218.

38. Lapidot, T., et al., *A cell initiating human acute myeloid leukaemia after transplantation into SCID mice.* Nature, 1994. **367**(6464): p. 645-8.

39. Eckhoff, C., M.D. Collins, and H. Nau, *Human plasma all-trans-, 13-cis- and 13-cis-4-oxoretinoic acid profiles during subchronic vitamin A supplementation: comparison to retinol and retinyl ester plasma levels.* J Nutr, 1991. **121**(7): p. 1016-25.

40. Ryningen, A., et al., *In vivo biological effects of ATRA in the treatment of AML.* Expert Opin Investig Drugs, 2008. **17**(11): p. 1623-33.

41. Wang, Y., R. Zolfaghari, and A.C. Ross, *Cloning of rat cytochrome P450RAI (CYP26) cDNA and regulation of its gene expression by all-trans-retinoic acid in vivo.* Arch Biochem Biophys, 2002. **401**(2): p. 235-43.

42. Ozpolat, B., K. Mehta, and G. Lopez-Berestein, *Regulation of a highly specific retinoic acid-4-hydroxylase (CYP26A1) enzyme and all-trans-retinoic acid metabolism in human intestinal, liver, endothelial, and acute promyelocytic leukemia cells.* Leuk Lymphoma, 2005. **46**(10): p. 1497-506.

43. Adamson, P.C., et al., *Phase I trial and pharmacokinetic study of all-trans-retinoic acid administered on an intermittent schedule in combination with interferon-alpha2a in pediatric patients with refractory cancer.* J Clin Oncol, 1997. **15**(11): p. 3330-7.

44. Turhan, A.G., et al., *Highly purified primitive hematopoietic stem cells are PML-RARA negative and generate nonclonal progenitors in acute promyelocytic leukemia.* Blood, 1995. **85**(8): p. 2154-61.

45. Geria, A.N. and N.S. Scheinfeld, *Talarozole, a selective inhibitor of P450-mediated all-trans retinoic acid for the treatment of psoriasis and acne.* Curr Opin Investig Drugs, 2008. **9**(11): p. 1228-37.

46. Tobita, T., et al., *Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid.* Blood, 1997. **90**(3): p. 967-73.

47. Osanai, M. and M. Petkovich, *Expression of the retinoic acid-metabolizing enzyme CYP26A1 limits programmed cell death.* Mol Pharmacol, 2005. **67**(5): p. 1808-17.

CHAPTER 3

The Potential of Disease Clinical Status to Modulate Drug Metabolizing Enzymes in the Leukemic Bone Marrow Microenvironment

The potential of disease clinical status to modulate drug metabolizing enzymes in the leukemic bone marrow microenvironment

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3.1 Abstract

The bone marrow microenvironment contributes to drug resistance in acute myeloid leukemia (AML) and multiple myeloma (MM). We have shown that the critical drug metabolizing enzymes cytidine deaminase (CDA) and cytochrome P450 (CYP) 3A4 are highly expressed by bone marrow (BM) stroma, and play an important role in AML and MM resistance to chemotherapy. However, what factors influence the chemoprotective capacity of the BM microenvironment, specifically related to CYP3A4 and CDA expression, are unknown. In this study, we found the presence of AML cells decrease BM stromal expression of CYP3A4 and CDA, and this effect appears to be at least partially the result of cytokines secreted by AML cells. We also observed that stromal CYP3A4 expression is upregulated by drugs commonly used in AML induction therapy: cytarabine, etoposide, and daunorubicin. In addition, cytarabine also upregulated CDA expression. Moreover, the stromal-induced chemoprotection of AML induced by one drug cross-reacted to others, and was blocked by clarithromycin, a potent inhibitor of CYP3A4. Our data suggest that AML chemotherapy enhances microenviroment-mediated drug resistance by upregulation of drug metabolizing enzymes. These results further suggest a potential role for clinically targeting drug metabolizing enzymes in the microenviroment.

3.2 Introduction

Most patients with acute myeloid leukemia (AML) and other hematologic malignancies achieve complete remissions (CRs) with initial chemotherapy, but eventually relapse and die of their disease [1]. The mechanisms responsible for the resistance of minimal residual disease (MRD), the disease remaining in CR that leads to relapse, to therapy are also unclear. Emerging data suggest that the cancer stem cell (CSC) concept could explain why dramatic responses often fail to translate into cures[2, 3]; this concept hypothesizes that relapse in many cancers appears to result from rare cells with stem cell characteristics; these so-called CSCs are often biologically distinct from their progeny that form the bulk of the tumor, notably exhibiting substantially different sensitivity to drugs. In addition to intrinsic mechanisms of drug resistance in MRD, it is now clear that specialized microenvironments or niches play important roles in extrinsic drug resistance [1, 4-6]. Our group previously showed that bone marrow (BM) stomal cells protect normal human hematopoietic stem cells (HSCs) and AML cells from the pro-differentiating effects of retinoic acid by expressing the retinoid-inactivating enzyme, cytochrome P450 (CYP)26 [7, 8]. We also found that stromal CYP3A4 similarly protected AML and multiple myeloma (MM) cells from various chemotherapeutic agents [9, 10]. However, the exact mechanisms responsible for regulating stromal CYPs are unclear.

It is known that the leukemic bone marrow is a pro-inflammatory, cytokine rich environment [11] and many of these factors, such as IL6, play important roles in AML biology [12-15]. Cytokines and inflammation, especially related to and cancer, have been

shown to suppress hepatic and intestinal CYP levels [16-20]. For the initial treatment of newly-diagnosed AML patients, the "7+3" regimen, which combine a seven-day continuous intravenous infusion of cytarabine (ara-C) (100 or 200 mg/m^2 per day) with a short infusion of an anthracycline given on days one through three, remains the most commonly used regimen. Etoposide is another agent used in many induction regimens [21, 22]. All three of these agents are substrates for CYP3A4 [23, 24], and ara-C is also inactivated by cytidine deaminase (CDA) [25-27]. Chemotherapeutics can induce CYP3A4 activity in human liver cells [28]. Thus, the clinical status of the AML and its treatment could theoretically influence the expression of CYP3A4 and CDA. Accordingly, we hypothesized that effects of tumor burden and chemotherapy on the tumor microenvironment could play a role in why complete responses translate into cures in only a fraction of patients. In this study, we find that the BM stromal expression of CYP3A4 and CDA is influenced by the status of the AML and its treatment, and that clinically targeting drug metabolizing enzymes in the microenviroment holds promise.

3.3 Materials and Methods

3.3.1 Cell lines

The human fetal bone marrow stroma cell line F/STRO was a kind gift from Dr. Pierre Marie, and was cultured in DMEM (Gibco, Rockville, MD, USA) with 10% fetal calf serum (FCS) (Sigma-Aldrich), 100µg/mL penicillin-streptomycin (Gibco), and 2 mM L-glutamine (Life Technologies), as previously described[29]. The human AML cell line

HL-60 [30] was cultured in RPMI 1640 (Gibco) with 10% FCS, 100μg/mL penicillin-streptomycin (Gibco), and 2 mM L-glutamine (Life Technologies). The M2 AML cells Kasumi-1[31] were cultured in RPMI 1640 + 20% FCS and the NPM1 mutated OCI-AML3 cells[32] were cultured in minimum essential media (α-MEM) (Corning Cellgro) with 20% FCS, 2 mM L-glutamine and 100 μg/mL penicillin-streptomycin.

3.3.2 Isolation of primary bone marrow stroma

Primary bone marrow stroma cells were derived from normal bone marrow donors granting informed consent as approved by the Johns Hopkins Medical Institutes' Institutional Review Board, as we have previously described[33]. Briefly, mononuclear cells isolated from bone marrow of normal volunteers were cultured in FBMD1 media [IMDM media (Gibco) supplemented with 15% FBS (Sigma-Aldrich), 5% Horse serum (Sigma-Aldrich), 100μg/mL penicillin-streptomycin (Gibco), and 10^{-4} M β-mercaptoethanol (Sigma-Aldrich)][34] at 33˚C in 5%CO_2 overnight. The next day, media and cells in suspension were removed and the attached cells were washed twice with phosphate-buffered saline (PBS) (Gibco), fresh FBMD1 media was added to the flask and they were placed back 33˚C in 5% CO2. Half of the media was replaced weekly until an adherent monolayer has formed. At that time, the cells were dissociated using Trypsin (Gibco) and they were either used for further experiments or cryopreserved. The passage number of the cells was recorded with original cells labeled as P1. Experiments presented in this paper were performed using bone marrow stroma at passages 2-4.

3.3.3 Co-culture system

For co-culture conditions, 6-well plates (Sigma) were coated with 0.1% Gelatin (Sigma) in PBS for 20 min. at 37˚C. Gelatin solution was removed and stroma cells were seeded at a density of $20x10^4$ cells/well and cultured until a confluent monolayer was obtained. Subsequently, $2.5x10^4$ HL-60, Kasumi-1, and OCI/AML3 cells were plated per well. The cultures were treated with or without 10^{-6}M ara-C (Sigma) or 10^{-7}M daunorubicin (Selleckchem, TX, USA) as well as 10^{-6}M etoposide (Sigma) for 72h.

3.3.4 Conditioned medium

$7x10^4$ HL-60, Kasumi-1, and OCI/AML3 cells were cultured under conditions mentioned previously for 72h. The cells were centrifuged at 1200rpm for 5min and the supernatant was added onto monolayer of FSTRO cells in 6-well plates as previously mentioned.

3.3.5 Chemotherapy drug treatment

6-well plates (Sigma) were coated with 0.1% Gelatin (Sigma) in PBS for 20 min. at 37˚C. Gelatin solution was removed and stroma cells were seeded at a density of $20x10^4$ cells/well and cultured until a confluent monolayer was obtained. 10^{-6}M ara-C (Sigma), 10^{-7}M daunorubicin (Selleckchem), and 10^{-6}M VP-16 (Sigma) were added to stroma cells for 72h.

3.3.6 Colony forming unit (CFU-C)

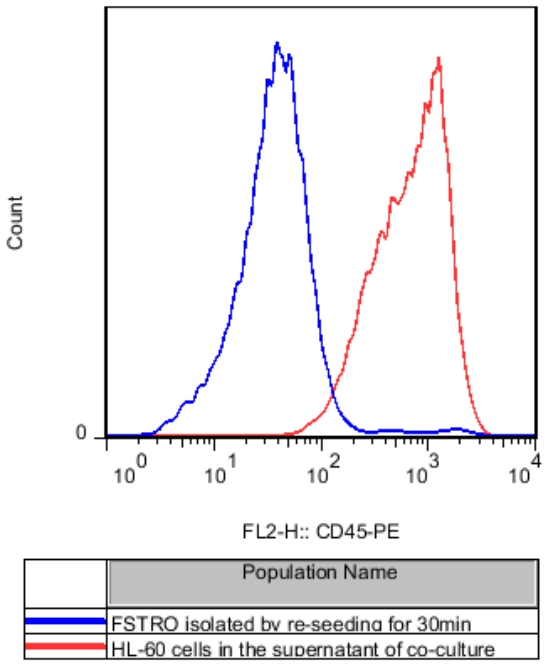
Clonogenic growth of AML cell lines was evaluated as we previously described [35, 36]. Briefly, previously treated cells were removed from the plate and washed with PBS to remove the respective drug. Cells were then counted using Trypan blue and plated 1 mL

1.2% methylcellulose (Sigma-Aldrich), 30% bovine serum albumin (BSA) (Sigma-Aldrich), 10^{-4} M β-mercaptoethanol (Sigma-Aldrich), and 2 mM L-glutamine (Gibco). Samples were plated in duplicate onto 35-mm^2 tissue culture dishes and incubated in a humidified atmosphere at 37°C and 5% CO_2. Colonies consisting of more than 40 cells were scored at 10-15 days using an inverted microscope.

3.3.7 RNA isolation

F/STRO and primary BM stroma in culture were dissociated using 0.05% Trypsin (Gibco) and collected for total RNA extraction using RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. For the F/STRO and primary BM stroma co-cultured with AML cells, all the cells attached to the plate were dissociated using 0.05% Trypsin (Gibco) after the supernatant containing part of the AML cells was washed off. The attached cells were re-seeded to the plate and incubated for 30min at 37 °C. Then the AML cells in the supernatant were washed off, and the adherent stroma cells were harvested using 0.05% Trypsin and used in RNA extraction later using RNeasy Mini kit (QIAGEN). The purity of this method separating stroma cells from co-culture is confirmed (Supplementary Figure 1).

Supplementary Figure 3.1. Purity of the method separating stroma cells from co-culture with AML cells by re-seeding to culture plate for 30 minutes.



Two populations of cells were separated from the co-culture system and showed by Flow Cytometry. The red line indicating the HL-60 AML cells taken from the supernatant of the co-culture plate. The blue line indication the F/STRO cells isolated by re-seeding all the attached cells to the plate for 30 min and harvest the adherent cells.

3.3.8 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

cDNA was synthesized by reverse transcription using the iScript cDNA synthesis kit (BIO-RAD, Hercules, CA). The sequences of the CYP3A4 primers were obtained from Integrated DNA Technologies (IDT) at Johns Hopkins Medical Institute DNA Analysis Facility, and are as follows: 5′-GCCTGGTGCTCCTCTATCTA-3′ (sense) and 5′-GGCTGTTGACCATCATAAAAG-3′ (anti-sense). The primers were designed for the amplification of a specific CYP3A4 DNA product, which spanned 3 introns of the CYP3A4 gene and covers both transcript variant 1 and 2 of CYP3A4. The sequences of the CDA primers are as follows: 5′-ATCGCCAGTGACATGCAAGA-3′ (sense) and 5′-GTACCATCCGGCTTGGTCAT-3′ (anti-sense). GAPDH was used as an endogenous control. The primers of GPADH are as follows: 5′-ACCCAGAAGACTGTGGATGG-3′ (sense) and 5′- TCTAGACGGCAGGTCAGGTC-3′ (anti-sense). qPCR was performed with an Bio-Rad CFX96^{TM} Real-Time PCR Detection System (Bio-Rad, Berkeley, CA) and Puregreen lo-ROX qPCR kit (Nextdayscience, Rockville, MD), in accordance with the manufacturer's protocol (a denaturation stage at 95 °C for 2 min; 40 cycles of 5 s at 95 °C, and 40 cycles of 30 s at 60 °C).

3.3.9 Statistical analysis

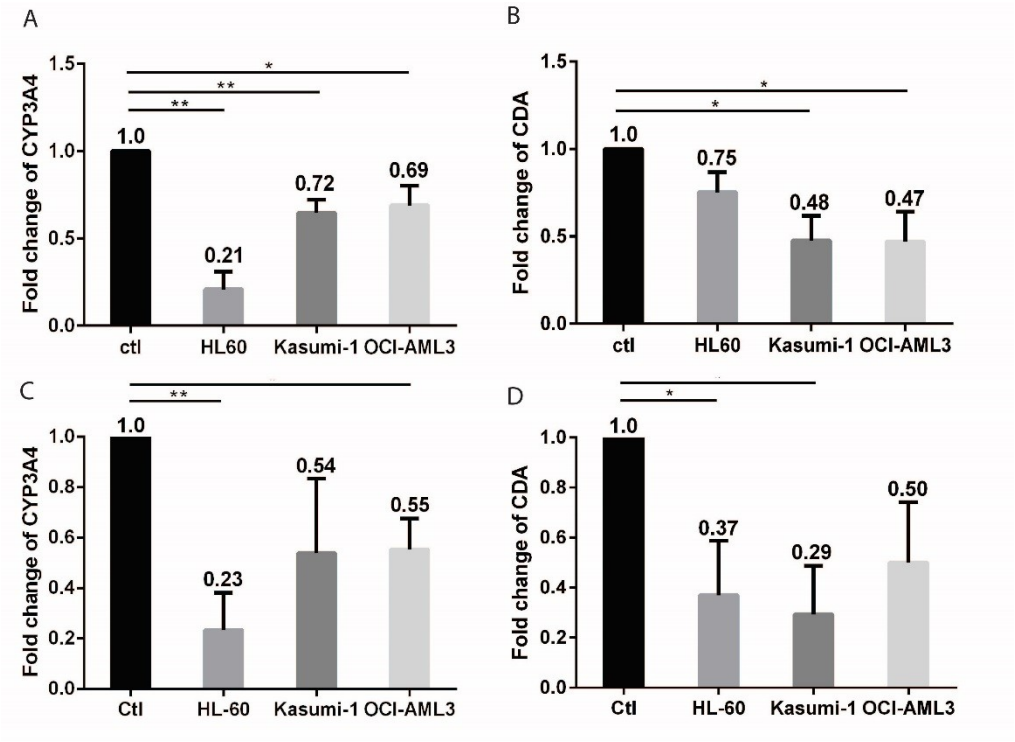
Statistical analysis was performed by using two-tail unpaired student t test to compare the averages of two groups and calculate the p value.

3.4 Results

3.4.1 Active AML generates a CYP3A4 and CDA low environment.

We previously showed that CYP3A4, other CYPs, and CDA were all highly expressed in BM stroma, but not AML and MM cells [9]. To model the BM stroma of AML patients, HL-60, Kasumi-1 and OCI-AML3 cells were co-cultured with human normal primary BM stroma for 72 hours, and the expression of CYP3A4 and CDA in stroma cells was assessed by quantitative reverse transcription PCR (RT-qPCR). All three AML lines significantly suppressed the expression of both CYP3A4 and CDA in both primary BM stroma (Figure 1A and 1B, respectively) and the human BM stomal line F/STRO (Figure 1C and 1D, respectively).

Figure 3.1. Effect of AML cells and their culture supernatant to BM MSCs in expression of CYP3A4 and CDA

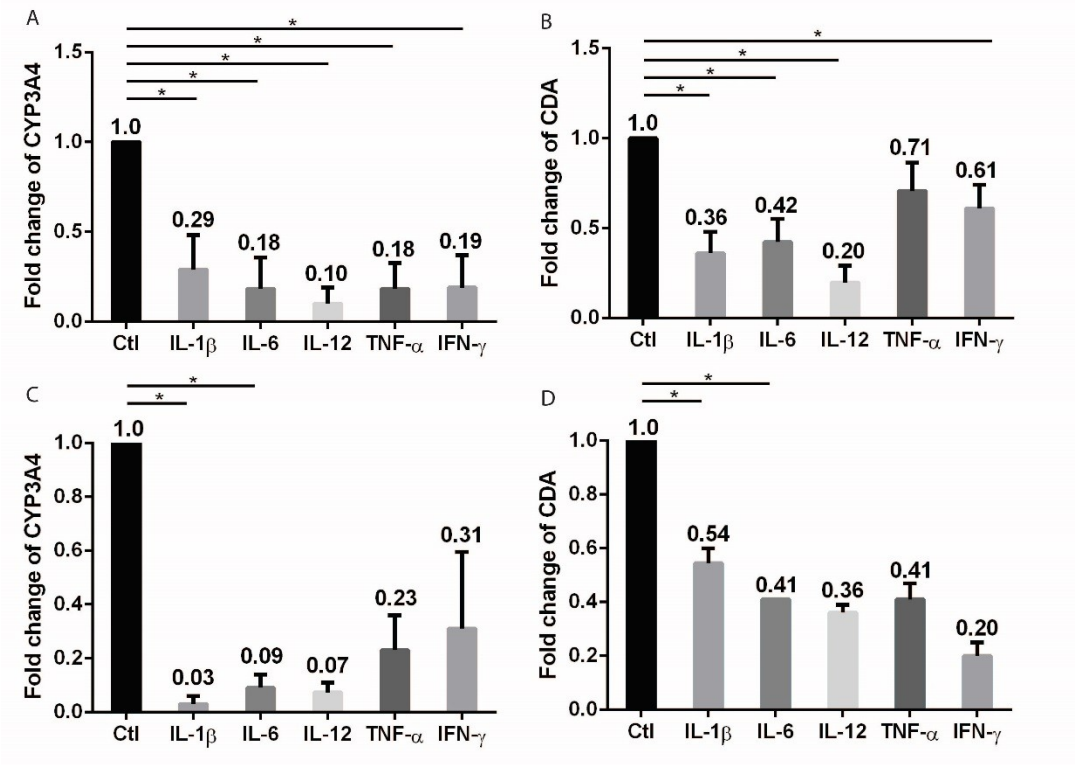


A-B. Relative quantification of CYP3A4 **(A)** and CDA **(B)** mRNA expression in F/STRO after treatment of medium cultured HL-60, Kasumi-1 and OCI-AML3 for 72 hours. **C-D.** Relative quantification of CYP3A4 **(C)** and CDA **(D)** mRNA expression in primary human BM MSCs from three different healthy BM donors, after co-cultured with AML cell lines HL-60, Kasumi-1 and OCI-AML3 for 72 hours. CYP3A4 and CDA expression was normalized to GAPDH, and relative quantification was calculated using ΔΔCT. Expression of CYP3A4 and CDA are presented relative to non-treatment control. Results show mean ± SEM of 3 independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$.

3.4.2 Cytokines associated with AML downregulate CYP3A4 and CDA

It is well-documented that the BM during active AML is a pro-inflammatory environment, associated with aberrant cytokine signaling [13, 15]. Moreover, inflammatory cytokines, such as IL-1, IL-6 and TNF-α can downregulate hepatic and intestinal CYP3A4 levels [18, 19]. Cytokines known to be elevated in AML, IL-1β [37], IL-6 [14], IL-12 [13], TNF-α [13, 38], and INF-γ [39] were studied for their ability to modulate CYP3A4 and CDA expression in BM stromal cells. After 72 hours of incubation with these cytokines, CYP3A4 and CDA mRNA expression was measured in BM stromal cells by RT-qPCR. All cytokines tested significantly suppressed the expression of both CYP3A4 and CDA in both human BM stomal line F/STRO (Figure 2A and 2B, respectively) and the primary BM stroma (Figure 2C and 2D, respectively).

Figure 3.2. Effect of cytokines enriched in AML to BM MSCs in expression of CYP3A4 and CDA

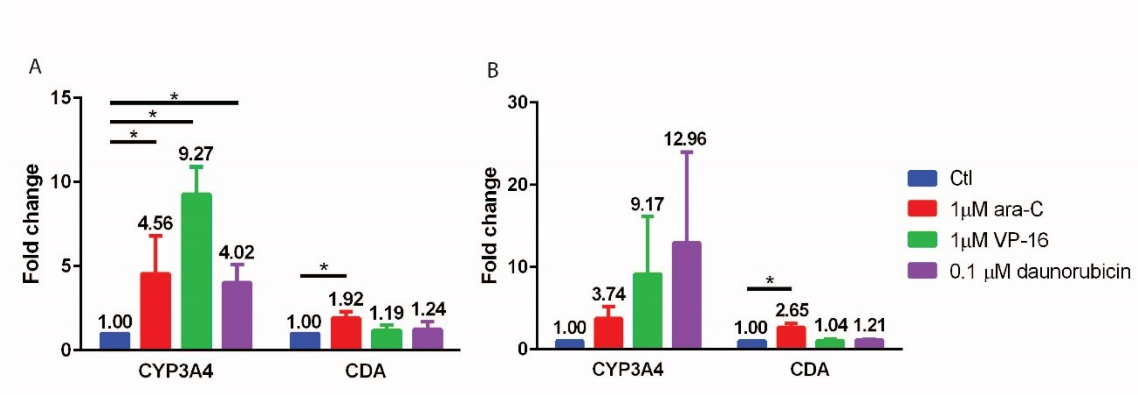


A-B. Effects of IL-1β, IL-6, IL-12, TNF-α, and IFN-γ (10ng/ml for 72 hrs) on the CYP3A4 **(A)** and CDA **(B)** mRNA expression in primary human BM MSCs from three different healthy BM donors. **C-D.** Effects of IL-1β, IL-6, IL-12, TNF-α, and IFN-γ (10ng/ml for 72 h) on the CYP3A4 **(C)** and CDA **(D)** mRNA expression in F/STRO. Results show mean ± SEM, n = 3, *$p < 0.01$.

3.4.3 CYP3A4 mRNA expression in human BM stroma cells is upregulated by ara-C, etoposide, and daunorubicin, while CDA is upregulated only by ara-C

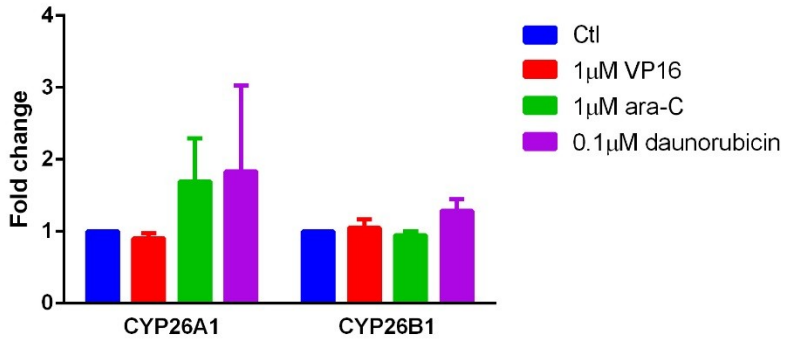
The most commonly-used chemotherapy drugs for remission induction in AML were assessed for the ability to induce the expression drug metabolizing enzymes in BM stroma, as they have been reported to do in liver [28]. Ara-C, etoposide, and daunorubicin all upregulated CYP3A4 expression of F/STRO stromal cells, while only ara-C upregulated CDA (Figure 3A). Similar results were also seen in primary BM stroma (Figure 3B). To further confirm the specificity of the drug-induced upregulation, CYP26A1 and CYP26B1, enzymes involved in retinoid but not chemotherapy inactivation, were measured and found not to be changed significantly upregulated by ara-C, etoposide, or daunorubicin (Supplementary Figure 2).

Figure 3.3. Effect of induction chemotherapy drugs in AML to BM MSCs in expression of CYP3A4 and CDA



A. Effects of 1μM ara-C, 1μM VP-16, and 0.1μM daunorubicin treatment for 72 hours on the mRNA expression levels of CYP3A4 and CDA in F/STRO. **B.** Effects of 1μM ara-C, 1μM VP-16, and 0.1μM daunorubicin treatment for 72 hours on the mRNA expression levels of CYP3A4 and CDA in primary human BM MSCs from three different healthy BM donors. Data are presented as mean ± SEM, n = 3, $^{**}p < 0.01$, $^{*}p < 0.05$.

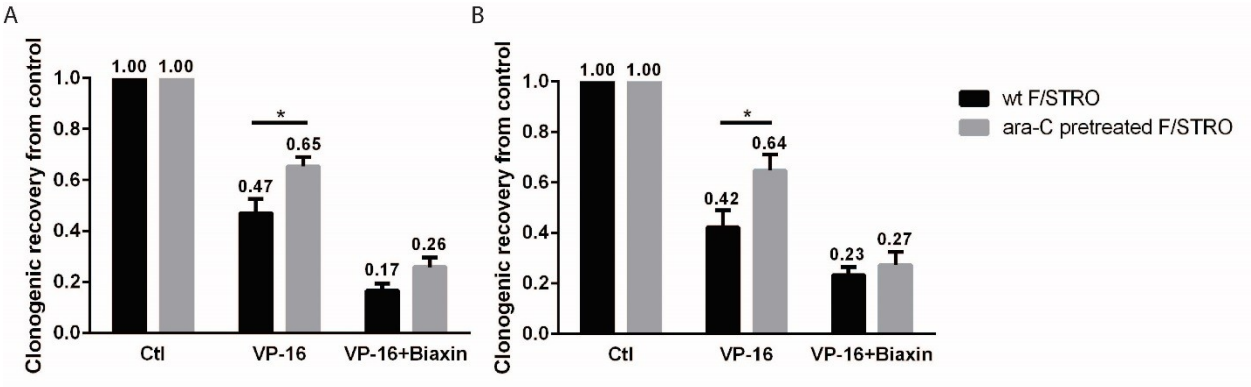
Supplementary Figure 3.2. Effect of induction chemotherapy drugs in AML to BM MSCs in expression of CYP26A1 and CYP26B1



Effects of 1μM ara-C, 1μM VP-16, and 0.1μM daunorubicin treatment for 72 hours on the mRNA expression levels of CYP26A1 and CYP26B1 in primary human BM MSCs from three different healthy BM donors. Data are presented as mean ± SEM, n ≥ 3.

To determine the functional effect of upregulation of drug metabolizing enzymes, the effects of drug treatment of BM stroma on AML sensitivity was assessed. AML induction chemotherapy is generally given over 7-10 days usually with 2-3 drugs often given sequentially[40, 41] Accordingly, F/STRO BM stromal cells were pre-incubated with ara-C for 72 hours, and then after removing the drug, co-cultured with two AML cell lines (HL-60 and OCI-AML3) with or without etoposide**.** Preincubation of stomal cells with ara-C further protected both the HL-60 cells (Figure 4A) and OCI-AML3 cells (Figure 4B) against etoposide. Clarithromycin (Biaxin), a potent CYP3A4 inhibitor [9, 42, 43], reversed the ara-C pretreatment protection (Figures 4A, B).

Figure 3.4. Role of stromal CYP3A4 in ara-C induced stroma-mediated resistance of AML cells to VP-16 treatment



Effects of VP-16 (1 μM for 72 h) on clonogenic activity of HL-60 **(A)** and OCI-AML3 **(B)** AML cells co-cultured with F/STRO BM stroma and ara-C pretreated F/STRO. **A.** Treatment of HL-60 cells co-cultured with wt F/STRO with VP-16 resulted in 47 ± 6%, while HL-60 cells co-cultured with ara-C pretreated F/STRO resulted in 65 ± 4% clonogenic activity compared to control cultures. Treatment with 1 μM Biaxin reversed the protective effect of both wt F/STRO and ara-C pretreated F/STRO to HL-60 cells, resulted in 17 ± 3% and 26 ± 4% clonogenic activity, respectively. **B.** Treatment of OCI-AML3 cells co-cultured with wt F/STRO with VP-16 resulted in 42 ± 7%, while co-cultured with ara-C pretreated F/STRO resulted in 64 ± 6% clonogenic activity compared to control cultures. Treatment with 1 μM Biaxin reversed the protective effect of both wt F/STRO and ara-C pretreated F/STRO to OCI-AML3 cells again VP-16, resulted in 23 ± 3% and 27 ± 5% clonogenic activity compared to control, respectively. Data are presented as mean ± SEM of at least three independent experiments. *$p < 0.05$.

3.5 Discussion

Previously, our group showed that expression of CYP enzymes appears to be at least partly responsible for the well-recognized ability of BM stroma to protect AML and MM from chemotherapy [7, 9]. The BM during leukemia therapy is a dynamic environment with changes related to treatment and tumor burden, and it is well-established that cancer inflammation will modulate CYP expression in the liver [20]. We found that both AML cells as well as inflammatory cytokines that are elevated during AML, decrease of CYP3A4 and CDA gene expression level in BM stromal, similar to the effect of inflammatory cytokines on liver CYP3A4 expression. In addition, we also observed the most commonly used drugs for newly-diagnosed AML led to an induction of CYP3A4 and CDA expression in BM stromal cells. Our findings suggest chemotherapy drug metabolizing enzymes in the BM MSCs of AML patients can be modulated by the clinical status of bone marrow and chemotherapy drugs, and provide a basis for additional studies to address the extent to which these observed changes contribute to leukemogenesis or response to treatment.

Accordingly, our data suggest that the induction therapy of AML makes the BM microenvironment a more drug resistance one. Along with chemotherapy's ability to induce stroma drug-metabolizing enzymes, the leukemic burden is decreased and the BM microenvironment returns to relatively normal state. Overexpression of cytokines in leukemia patients declines with remission attainment [14], and our data suggest that both the elimination of leukemia and normalization of cytokines should further upregulate CYP3A4 and CDA in the BM.

These data suggest that the BM microenvironment may plan an important role in the relative drug resistance of MRD. Further, our findings suggest a potential role of targeting microenvironment drug detoxifying agents in combination with chemotherapy. Inhibition of CYP3A4 and CDA in patients at diagnosis and primary refractory disease, may render their AML susceptible to chemotherapy. Also, inhibition of CYP3A4 and CDA during consolidation therapy, may result in improved elimination of MRD.

3.6 References

1. Estey, E. and H. Dohner, *Acute myeloid leukaemia.* Lancet, 2006. **368**(9550): p. 1894-907.
2. Ghiaur, G., J. Gerber, and R.J. Jones, *Concise review: Cancer stem cells and minimal residual disease.* Stem Cells, 2012. **30**(1): p. 89-93.
3. Yanagisawa, B., et al., *Translating leukemia stem cells into the clinical setting: Harmonizing the heterogeneity.* Exp Hematol, 2016. **44**(12): p. 1130-1137.
4. Garrido, S.M., et al., *Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5).* Exp Hematol, 2001. **29**(4): p. 448-57.
5. Hao, M., et al., *Bone marrow stromal cells protect myeloma cells from bortezomib induced apoptosis by suppressing microRNA-15a expression.* Leuk Lymphoma, 2011. **52**(9): p. 1787-94.

6. Nefedova, Y., T.H. Landowski, and W.S. Dalton, *Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms.* Leukemia, 2003. **17**(6): p. 1175-82.

7. Su, M., et al., *All-Trans Retinoic Acid Activity in Acute Myeloid Leukemia: Role of Cytochrome P450 Enzyme Expression by the Microenvironment.* PLoS One, 2015. **10**(6): p. e0127790.

8. Ghiaur, G., et al., *Regulation of human hematopoietic stem cell self-renewal by the microenvironment's control of retinoic acid signaling.* Proc Natl Acad Sci U S A, 2013. **110**(40): p. 16121-6.

9. Alonso, S., et al., *Human bone marrow niche chemoprotection mediated by cytochrome P450 enzymes.* Oncotarget, 2015. **6**(17): p. 14905-12.

10. Alonso, S., et al., *Hedgehog and retinoid signaling alters multiple myeloma microenvironment and generates bortezomib resistance.* J Clin Invest, 2016. **126**(12): p. 4460-4468.

11. Kupsa, T., J.M. Horacek, and L. Jebavy, *The role of cytokines in acute myeloid leukemia: a systematic review.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2012. **156**(4): p. 291-301.

12. Bruserud, O., et al., *Effects of hematopoietic growth factors on interleukin 6 secretion by blast cells derived from acute myelogenous leukemia patients.* Acta Haematol, 1996. **96**(3): p. 155-61.

13. Sanchez-Correa, B., et al., *Cytokine profiles in acute myeloid leukemia patients at diagnosis: survival is inversely correlated with IL-6 and directly correlated with IL-10 levels.* Cytokine, 2013. **61**(3): p. 885-91.

14. Hsu, H.C., et al., *Circulating levels of thrombopoietic and inflammatory cytokines in patients with acute myeloblastic leukemia and myelodysplastic syndrome.* Oncology, 2002. **63**(1): p. 64-9.

15. Van Etten, R.A., *Aberrant cytokine signaling in leukemia.* Oncogene, 2007. **26**(47): p. 6738-49.

16. Machavaram, K.K., et al., *A physiologically based pharmacokinetic modeling approach to predict disease-drug interactions: suppression of CYP3A by IL-6.* Clin Pharmacol Ther, 2013. **94**(2): p. 260-8.

17. Aitken, A.E., T.A. Richardson, and E.T. Morgan, *Regulation of drug-metabolizing enzymes and transporters in inflammation.* Annu Rev Pharmacol Toxicol, 2006. **46**: p. 123-49.

18. Morgan, E.T., *Impact of infectious and inflammatory disease on cytochrome P450-mediated drug metabolism and pharmacokinetics.* Clin Pharmacol Ther, 2009. **85**(4): p. 434-8.

19. Bertilsson, P.M., P. Olsson, and K.E. Magnusson, *Cytokines influence mRNA expression of cytochrome P450 3A4 and MDRI in intestinal cells.* J Pharm Sci, 2001. **90**(5): p. 638-46.

20. Kacevska, M., et al., *Inflammation and CYP3A4-mediated drug metabolism in advanced cancer: impact and implications for chemotherapeutic drug dosing.* Expert Opin Drug Metab Toxicol, 2008. **4**(2): p. 137-49.

21. Kolitz, J.E., et al., *Dose escalation studies of cytarabine, daunorubicin, and etoposide with and without multidrug resistance modulation with PSC-833 in untreated adults with acute myeloid leukemia younger than 60 years: final*

induction results of Cancer and Leukemia Group B Study 9621. J Clin Oncol, 2004. **22**(21): p. 4290-301.

22. Kolitz, J.E., et al., *P-glycoprotein inhibition using valspodar (PSC-833) does not improve outcomes for patients younger than age 60 years with newly diagnosed acute myeloid leukemia: Cancer and Leukemia Group B study 19808.* Blood, 2010. **116**(9): p. 1413-21.
23. Fujita, K., *Cytochrome P450 and anticancer drugs.* Curr Drug Metab, 2006. **7**(1): p. 23-37.
24. Colburn, D.E., et al., *In vitro evaluation of cytochrome P450-mediated drug interactions between cytarabine, idarubicin, itraconazole and caspofungin.* Hematology, 2004. **9**(3): p. 217-21.
25. Yue, L., et al., *A functional single-nucleotide polymorphism in the human cytidine deaminase gene contributing to ara-C sensitivity.* Pharmacogenetics, 2003. **13**(1): p. 29-38.
26. Ciccolini, J., et al., *Integrating pharmacogenetics into gemcitabine dosing--time for a change?* Nat Rev Clin Oncol, 2011. **8**(7): p. 439-44.
27. Kirch, H.C., et al., *Recombinant gene products of two natural variants of the human cytidine deaminase gene confer different deamination rates of cytarabine in vitro.* Exp Hematol, 1998. **26**(5): p. 421-5.
28. Goldstein, I., et al., *Chemotherapeutic agents induce the expression and activity of their clearing enzyme CYP3A4 by activating p53.* Carcinogenesis, 2013. **34**(1): p. 190-8.

29. Ahdjoudj, S., et al., *Reciprocal control of osteoblast/chondroblast and osteoblast/adipocyte differentiation of multipotential clonal human marrow stromal F/STRO-1(+) cells.* J Cell Biochem, 2001. **81**(1): p. 23-38.

30. Collins, S.J., R.C. Gallo, and R.E. Gallagher, *Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture.* Nature, 1977. **270**(5635): p. 347-9.

31. Asou, H., et al., *Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation.* Blood, 1991. **77**(9): p. 2031-6.

32. Quentmeier, H., et al., *Cell line OCI/AML3 bears exon-12 NPM gene mutation-A and cytoplasmic expression of nucleophosmin.* Leukemia, 2005. **19**(10): p. 1760-7.

33. Ghiaur, G., et al., *Regulation of human hematopoietic stem cell self-renewal by the microenvironment's control of retinoic acid signaling.* Proc Natl Acad Sci U S A, 2013.

34. Breems, D.A., et al., *Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay.* Leukemia, 1994. **8**(7): p. 1095-104.

35. Matsui, W.H., et al., *The role of growth factors in the activity of pharmacological differentiation agents.* Cell Growth Differ, 2002. **13**(6): p. 275-83.

36. Bedi, A., et al., *BCR-ABL-mediated inhibition of apoptosis with delay of G2/M transition after DNA damage: a mechanism of resistance to multiple anticancer agents.* Blood, 1995. **86**(3): p. 1148-58.

37. Griffin, J.D., et al., *Secretion of interleukin-1 by acute myeloblastic leukemia cells in vitro induces endothelial cells to secrete colony stimulating factors.* Blood, 1987. **70**(4): p. 1218-21.

38. Tsimberidou, A.M., et al., *The prognostic significance of cytokine levels in newly diagnosed acute myeloid leukemia and high-risk myelodysplastic syndromes.* Cancer, 2008. **113**(7): p. 1605-13.

39. Kornblau, S.M., et al., *Recurrent expression signatures of cytokines and chemokines are present and are independently prognostic in acute myelogenous leukemia and myelodysplasia.* Blood, 2010. **116**(20): p. 4251-61.

40. Bishop, J.F., et al., *Intensified induction chemotherapy with high dose cytarabine and etoposide for acute myeloid leukemia: a review and updated results of the Australian Leukemia Study Group.* Leuk Lymphoma, 1998. **28**(3-4): p. 315-27.

41. Thomas, X., et al., *Granulocyte-macrophage colony-stimulating factor (GM-CSF) to increase efficacy of intensive sequential chemotherapy with etoposide, mitoxantrone and cytarabine (EMA) in previously treated acute myeloid leukemia: a multicenter randomized placebo-controlled trial (EMA91 Trial).* Leukemia, 1999. **13**(8): p. 1214-20.

42. Niemi, M., P.J. Neuvonen, and K.T. Kivisto, *The cytochrome P4503A4 inhibitor clarithromycin increases the plasma concentrations and effects of repaglinide.* Clin Pharmacol Ther, 2001. **70**(1): p. 58-65.

43. Spicer, S.T., et al., *The mechanism of cyclosporine toxicity induced by clarithromycin.* Br J Clin Pharmacol, 1997. **43**(2): p. 194-6.

44. Schepers, K., et al., *Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche.* Cell Stem Cell, 2013. **13**(3): p. 285-99.

45. Chandran, P., et al., *Mesenchymal stromal cells from patients with acute myeloid leukemia have altered capacity to expand differentiated hematopoietic progenitors.* Leuk Res, 2015. **39**(4): p. 486-93.

46. Jover, R., et al., *Down-regulation of human CYP3A4 by the inflammatory signal interleukin-6: molecular mechanism and transcription factors involved.* FASEB J, 2002. **16**(13): p. 1799-801.

47. Martinez-Jimenez, C.P., et al., *Transcriptional regulation of the human hepatic CYP3A4: identification of a new distal enhancer region responsive to CCAAT/enhancer-binding protein beta isoforms (liver activating protein and liver inhibitory protein).* Mol Pharmacol, 2005. **67**(6): p. 2088-101.

48. Fabiani, E., et al., *Polymorphisms of detoxification and DNA repair enzymes in myelodyplastic syndromes.* Leuk Res, 2009. **33**(8): p. 1068-71.

49. Binato, R., et al., *The molecular signature of AML mesenchymal stromal cells reveals candidate genes related to the leukemogenic process.* Cancer Lett, 2015. **369**(1): p. 134-43.

50. Li, C.Y., et al., *Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy.* Stem Cell Res Ther, 2015. **6**: p. 55.

51. Moll, G., et al., *Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties?* Stem Cells, 2014. **32**(9): p. 2430-42.

52. Marquez-Curtis, L.A., et al., *Mesenchymal stromal cells derived from various tissues: Biological, clinical and cryopreservation aspects.* Cryobiology, 2015. **71**(2): p. 181-97.

CHAPTER 4

Conclusions and Future Directions

4.1 Advances in our knowledge of CYP26 in BM microenvironment of hematological malignancies

4.1.1 CYP26 alters multiple myeloma microenvironment and generates bortezomib resistance

Since the publication of our work on BM stromal CYP26 caused drug resistance in AML (Chapter 2), there had been significant advances in our knowledge of the biology of CYP26 in BM microenvironment and CYP26 targeting treatments in hematological malignancies. Alonso, S et al [1] in our group has recently shown that an RA-low environment induced by stromal CYP26 is responsible for maintaining a B cell–like, bortezomib (BTZ) -resistant phenotype in multiple myeloma (MM) cells. Despite being broadly studied in many hematological malignancies, the differentiation therapy using retinoids has proved beneficial only in patients with APL [2]. Our studies in Chapter 2 suggest that CYP26 expression by BM stromal cells may explain the lack of a clinical benefit of natural retinoids, despite their in vitro activity. Recent studies have highlighted the efficacy of CYP-resistant synthetic retinoids in differentiating therapy. For instance, AM80 differentiates FMS-like tyrosine kinase 3/internal tandem duplication (FLT3/ITD) AML cells and increase their sensitivity to FLT3 inhibitors [3]. Such strategy of bypassing stromal CYP26 could expand the clinical effectiveness of retinoid therapy. Alonso, S et al proved that directly inhibiting CYP26 or bypassing stromal protection via a CYP26-resistant retinoid rescues plasma cells (PCs) differentiation and BTZ sensitivity. Furthermore, we described a bidirectional crosstalk, in which paracrine Hedgehog secreted by MM cells reinforces a protective niche via an increase in the ability of BM

stroma to inactivate RA. These data suggested that modulation of RA signaling, such as using CYP26-resistant retinoids, is a promising therapeutic strategy for overcoming BTZ resistance in the MM BM microenvironment.

4.1.2 Identification of drugs which overcome bone marrow stromal CYP26 in AML

Our group further unveiled the roles of ATRA signaling in the BM microenvironment and identified drugs which could overcome bone marrow stromal protection from ATRA induced differentiation in APL and non-APL cells. We have found in vitro that the synthetic retinoid AM80 [4] and IRX5183 (unpublished data) can overcome the protection provided AML cells by CYP26 in bone marrow stroma. AML cell lines from APL, NPM1 mutated and core binding factor leukemia (NB4, OCI/AML3, Kasumi respectively) were co-cultured with the mouse BM stroma cell line OP-9 and treated with ATRA, AM80 or IRX5183. Whereas stroma blocked upregulation of myeloid differentiation antigens and caused inhibition of clonogenic activity of AML cells by ATRA, both AM80 and IRX5183 induced differentiation in the presence or absence of stroma. These data support continued in vitro and in vivo experimentation with CYP resistant retinoids. We have developed a phase I clinical trial using IRX5183 in patients with relapsed and refractory AML. Given the success of retinoids in APL and their low toxicity profile, this may represent a significant advance in the treatment of AML.

In the meanwhile, we tested the impact of RARα and RARγ signaling on stromal CYP26 levels. The aforementioned synthetic retinoid AM80 is a strong RARα and weak RARγ

and RARβ agonist, IRX5183 is a specific RARα agonist, and ATRA is a pan-RAR agonist which was shown to be more selective for RARβ and RARγ with a higher binding affinity to these receptors at lower concentrations. By treating OP-9 BM stroma cells with these agents in serum free media, we observed that ATRA upregulated CYP26B1 at 24 hours followed by a slow drop towards initial level, while AM80 induced a constant upregulation of CYP26B1 through 48 hours. IRX5183 only had a modest upregulation throughout the 72 hours. Consistent with this, there is no upregulation of CYP26B1 in RARγ receptors knockdown human primary stroma, but there is upregulation of CYP26B1 in RARα receptor knockdown stroma. Even after 24 hours of ATRA treatment there is mainly upregulation of CYP26B1 in the RARα KD model. These results suggest that the activation of RARα is able to differentiate leukemia cells, while RARγ is dispensable for differentiation; and that the activation of RARγ causes up regulation of CYP26B1 and creates a more protective microenvironment (unpublished data).

4.2 Advances in our knowledge of CYP3A4 in BM microenvironment of hematological malignancies and drug resistance

4.2.1 CYP3A4 mediated chemoprotection in BM microenvironment of multiple myeloma and AML

Our initial studies [5, 6] demonstrated that normal and malignant stem cells are protected from the pro-differentiating effects of RA by BM microenvironment via CYP26. It is well known that the BM niche protects cancer cells not only from RA, but from virtually

any chemotherapy agent used in the clinic. Based on this, we proposed that the BM niche protects malignant cells from chemotherapy via drug inactivation. In despite the efficacy of chemotherapy to eliminate leukemic cells from the peripheral circulation, they survive within the BM niche, contributing to MRD and relapse. Indeed, the vast majority of detoxifying enzymes of the P450 superfamily are highly expressed by the BM niche at comparable levels to human hepatocytes [7]. Non-P450 metabolizing enzymes are also expressed in BM stroma, including cytidine deaminase. In contrast, expression of detoxifying enzymes within hematopoietic compartment is minimal.

As mentioned in Chapter 1, CYP3A4 is the most important P450 enzyme in the liver and is also expressed in the BM niche [8]. CYP3A4 metabolizes over 50% of therapeutic drugs, including bortezomib, used in in the treatment of MM. Despite achieving significant responses in patients, bortezomib does not completely eradicate MM cells from the BM. For this reason, we evaluated the role of stromal CYP3A4 on bortezomib resistance. Despite being highly sensitive to bortezomib, MM cells become resistant in the presence of BM mesenchymal cells. However, inhibition of stromal CYP3A4 by ketoconazole, or shRNA-mediated knockdown, rescues sensitivity to bortezomib [7]. This suggests that inactivation by the BM niche is at least partially responsible for bortezomib resistance in multiple myeloma.

Dexamethasone is another CYP3A4 target used in the treatment of MM, often in combination with lenalidomide, as part of the Rev/Dex (Revlimid®, dexamethasone) regimen [9]. Early observations suggested that the addition of the antibiotic

clarithromycin (used in Chapter 3), a potent CYP3A4 inhibitor, in patients with respiratory infections, potentiated multiple myeloma treatment. Subsequent studies dismissed any activity of clarithromycin as single-agent, but confirmed its clinical benefit when combined with dexamethasone [10, 11]. Moreover, even patients refractory to high dose dexamethasone or Rev/Dex respond to the addition of clarithromycin [12]. While different mechanisms of action against multiple myeloma have been proposed [13-15]. We showed that clarithromycin disrupts the BM niche barriers for drug delivery. Consistent with this, inhibition of CYP3A4 by clarithromycin or shRNA-mediated knockdown, restores dexamethasone concentration in stromal conditioned media, and overcomes stromal protection of MM cells against dexamethasone [7]. Furthermore, clarithromycin has no effect on dexamethasone's activity in the absence of BM mesenchymal cells, suggesting that it exclusively targets the bone marrow niche.

To extend these findings to other hematological malignancies, we examined the role of stromal CYP3A4 on AML resistance against etoposide (VP-16). Analogous to bortezomib treatment of multiple myeloma cells, AML blasts become refractory to etoposide treatment in the presence of BM mesenchymal cells, unless etoposide inactivation is prevented via CYP3A4 inhibition or knockdown [7]. Moreover, xenografted AML tumors containing wild-type BM mesenchymal cells are refractory to etoposide treatment, evidenced by continued exponential growth. However, tumors containing BM mesenchymal cells with CYP3A4 knockdown exhibit a sustained response to etoposide. This further confirms the role of the BM niche in regulating local drug concentrations during systemic chemotherapy.

4.2.1 CYP3A4 mediated chemoprotection in BM microenvironment of FLT3-AML

After our aforementioned discoveries of drug resistance mechanism mediated by CYP26 and CYP3A4 in BM microenvironment of AML and MM, we further explore whether CYP3A4 contributes to bone marrow mediated FLT3-AML protection from FLT3 inhibitors.

FLT3-AML takes approximately 1/3 of AML patients, and is associate with worse prognosis, higher relapse rate, and lower survival rate. It is characterized by mutations in FLT3 gene in leukemic cells, which is a promising target to treat FLT3-AML. In clinical trials, patients treated with FLT3 inhibitors show excellent clearance of peripheral blasts. However, the effect is minor in bone marrow, resulting in failure of leukemia eradication. Therefore, we hypothesize that stromal CYP3A4, by inactivating FLT3 inhibitors, contributes to bone marrow-mediated FLT3-AML protection from FLT3 inhibitors. Using colony formatting units (CFU) assay, we found that when co-culture FLT3-AML and BM MSCs with FLT3 inhibitor treatment, both knocking down CYP3A4 in MSCs and adding clarithromycin (a CYP3A4 inhibitor) repealed the protection effect of stroma. In mouse xenograft model, knocking down stromal CYP3A4 significantly restored the sensitivity of FLT3-AML tumors to the FLT3 inhibitor. Furthermore, measured by liquid chromatography-tandem mass spectrometry, the concentration of the FLT3 inhibitor in BM stroma culture media decreased in a time-dependent manner, whereas no decrease was detected when the CYP3A4 in stroma was knocked down (Unpublished data). Our

data revealed a drug resistance mechanism mediated by CYP3A4 and CYP3A4 in BM microenvironment of FLT3-AML. By metabolizing FLT3 inhibitors, stromal CYP3A4 creates an environment with lower FLT3 inhibitor concentration which is inefficient for leukemic growth inhibition. Combining FLT3 inhibitors with drug-metabolizing enzyme inhibitors may become a promising strategy to overcome drug resistance against FLT3-AML. Our results provide new insights to understanding bone marrow niche-derived drug resistance in hematological malignancies.

4.3 Advances in targeting stem cells clinically in AML

Indeed, since the increasing evidences of leukemia stem cells (LSCs) are responsible for recurrence and therapeutic resistance [16-19], targeting LSCs is becoming an attractive therapeutic strategy. There are finished or in progress studies using monoclonal antibody therapy targeting CD33+ cells [20-22], CLL-1+ cells [23], and CD123+ cells [24, 25] on AML patients. In addition to directly targeting stem cells in consider of their cell-intrinsic mechanism, studies targeting the LSCs niche to attenuate their cell-extrinsic protection to the LSCs are also ongoing. There has been a clinical trial for inhibition of CXCR4/CXL12 axis in relapsed and refractory AML [26]. Other adhesion molecules such as CD44 [27] and V-CAM [28] have also been targeted to overcome the microenvironment-mediated drug resistance in AML. Based on our discoveries of detoxifying effects of CYPs in the microenvironment [6, 7], our group have developed several clinical trials aimed at overcoming this potential mechanism for LSC resistance. The heterogeneity, clinical significance and clinical targets of LSCs has been nicely reviewed by our group recently [29].

4.4 Concluding remarks

Current strategies to overcome niche-mediated chemo-protection are majorly aimed at mobilizing malignant cells from their niche, by targeting adhesion molecules or chemokines. Pre-clinical studies have shown promising results of such strategies; however, this has not translated in increased patient cures. Furthermore, these approaches have not fully explain the niche enigma, and as a consequence, are unable to target the root cause of the problem: cancer stem cells within the BM niche.

We have investigated a novel mechanism in which cytochrome P450 enzymes in the BM niche produce a drug-free sanctuary by inactivating retinoic acid and chemotherapy agents, protecting normal and malignant stem cells. We have also found the factors influence the chemoprotective capacity of the BMT microenvironment, including presence of AML cells and the cytokines secreted by AML cells, and treatment of chemotherapy drugs which commonly used in AML induction therapy. Our findings suggest a potential role for clinically targeting drug metabolizing enzymes in the BM microenviroment. Targeting these drug metabolizing enzymes, while adjusting pharmacologic doses to maintain safe systemic concentrations, should disrupt these sanctuaries and target cancer cells within their microenvironment, with the ultimate goal of increasing cures in hematologic malignancies.

Cancer is a devastating disease, and AML is one of the deadliest malignant tumors, with 5-year survivals of 26.6% (NCI SEER Stat Fact Sheets, 2006-2012). A high relapse rate

for AML patients is still a major barrier to the long-term survival of these patients. While 5-year survival is still relatively low, it is important to make progress in the development of new agents and hopefully eliminate minimal residual disease. With continued research and identification of novel therapeutic targets, improved clinical regiment and clinical care, AML might one day become a more manageable malignancy like many breast cancers and prostate cancers now are.

4.4 References

1. Alonso, S., et al., *Hedgehog and retinoid signaling alters multiple myeloma microenvironment and generates bortezomib resistance.* J Clin Invest, 2016. **126**(12): p. 4460-4468.
2. Huang, M.E., et al., *Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia.* Blood, 1988. **72**(2): p. 567-72.
3. Ma, H.S., et al., *FLT3 Inhibition and Retinoid Signaling Overcome Stromal Protection to Target FLT3/ITD-Expressing Leukemia Stem Cells in the Bone Marrow Microenvironment.* Blood, 2015. **126**(23): p. 790-790.
4. Norsworthy, K.J., et al., *RAR-Alpha Targeting Compounds Overcome Bone Marrow (BM) Stromal Protection of AML By CYP26.* Blood, 2015. **126**(23): p. 2474-2474.
5. Ghiaur, G., et al., *Regulation of human hematopoietic stem cell self-renewal by the microenvironment's control of retinoic acid signaling.* Proc Natl Acad Sci U S A, 2013. **110**(40): p. 16121-6.

6. Su, M., et al., *All-Trans Retinoic Acid Activity in Acute Myeloid Leukemia: Role of Cytochrome P450 Enzyme Expression by the Microenvironment.* PLOS ONE, 2015. **10**(6): p. e0127790.

7. Alonso, S., et al., *Human bone marrow niche chemoprotection mediated by cytochrome P450 enzymes.* Oncotarget, 2015. **6**(17): p. 14905-12.

8. Fujita, K., *Cytochrome P450 and anticancer drugs.* Curr Drug Metab, 2006. **7**(1): p. 23-37.

9. Rajkumar, S.V., et al., *Combination therapy with lenalidomide plus dexamethasone (Rev/Dex) for newly diagnosed myeloma.* Blood, 2005. **106**(13): p. 4050-3.

10. Coleman, M., et al., *BLT-D (clarithromycin [Biaxin], low-dose thalidomide, and dexamethasone) for the treatment of myeloma and Waldenstrom's macroglobulinemia.* Leuk Lymphoma, 2002. **43**(9): p. 1777-82.

11. Stewart, A.K., et al., *Lack of response to short-term use of clarithromycin (BIAXIN) in multiple myeloma.* Blood, 1999. **93**(12): p. 4441.

12. Ghosh, N., et al., *Clarithromycin overcomes resistance to lenalidomide and dexamethasone in multiple myeloma.* Am J Hematol, 2014. **89**(8): p. E116-20.

13. Moriya, S., et al., *Macrolide antibiotics block autophagy flux and sensitize to bortezomib via endoplasmic reticulum stress-mediated CHOP induction in myeloma cells.* Int J Oncol, 2013. **42**(5): p. 1541-50.

14. Nakamura, M., et al., *Clarithromycin attenuates autophagy in myeloma cells.* Int J Oncol, 2010. **37**(4): p. 815-20.

15. Ohara, T., et al., *Antibiotics directly induce apoptosis in B cell lymphoma cells*

derived from BALB/c mice. Anticancer Res, 2004. **24**(6): p. 3723-30.

16. Gerber, J.M., et al., *A clinically relevant population of leukemic CD34(+)CD38(-) cells in acute myeloid leukemia.* Blood, 2012. **119**(15): p. 3571-7.

17. Gerber, J.M., et al., *Association of acute myeloid leukemia's most immature phenotype with risk groups and outcomes.* Haematologica, 2016. **101**(5): p. 607-16.

18. Pearce, D.J., et al., *AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML.* Blood, 2006. **107**(3): p. 1166-73.

19. Vergez, F., et al., *High levels of CD34+CD38low/-CD123+ blasts are predictive of an adverse outcome in acute myeloid leukemia: a Groupe Ouest-Est des Leucemies Aigues et Maladies du Sang (GOELAMS) study.* Haematologica, 2011. **96**(12): p. 1792-8.

20. Burnett, A.K. and U. Mohite, *Treatment of older patients with acute myeloid leukemia--new agents.* Semin Hematol, 2006. **43**(2): p. 96-106.

21. Majeti, R., *Monoclonal antibody therapy directed against human acute myeloid leukemia stem cells.* Oncogene, 2011. **30**(9): p. 1009-19.

22. Tsimberidou, A.M., et al., *Mylotarg, fludarabine, cytarabine (ara-C), and cyclosporine (MFAC) regimen as post-remission therapy in acute myelogenous leukemia.* Cancer Chemother Pharmacol, 2003. **52**(6): p. 449-52.

23. Zhao, X., et al., *Targeting C-type lectin-like molecule-1 for antibody-mediated immunotherapy in acute myeloid leukemia.* Haematologica, 2010. **95**(1): p. 71-8.

24. He, S.Z., et al., *A Phase 1 study of the safety, pharmacokinetics and anti-leukemic*

activity of the anti-CD123 monoclonal antibody CSL360 in relapsed, refractory or high-risk acute myeloid leukemia. Leuk Lymphoma, 2015. **56**(5): p. 1406-15.

25. Jin, L., et al., *Monoclonal antibody-mediated targeting of CD123, IL-3 receptor alpha chain, eliminates human acute myeloid leukemic stem cells.* Cell Stem Cell, 2009. **5**(1): p. 31-42.

26. Uy, G.L., et al., *A phase 1/2 study of chemosensitization with the CXCR4 antagonist plerixafor in relapsed or refractory acute myeloid leukemia.* Blood, 2012. **119**(17): p. 3917-24.

27. Gul-Uludag, H., et al., *Polymeric nanoparticle-mediated silencing of CD44 receptor in CD34+ acute myeloid leukemia cells.* Leuk Res, 2014. **38**(11): p. 1299-308.

28. Matsunaga, T., et al., *Combination therapy of an anticancer drug with the FNIII14 peptide of fibronectin effectively overcomes cell adhesion-mediated drug resistance of acute myelogenous leukemia.* Leukemia, 2008. **22**(2): p. 353-60.

29. Yanagisawa, B., et al., *Translating leukemia stem cells into the clinical setting: Harmonizing the heterogeneity.* Exp Hematol, 2016. **44**(12): p. 1130-1137.

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EDUCATION

B.S. in Medical Technology, Minor in Economics **2005-2009**

Peking University School of Medical Sciences, Beijing, China

- Laboratory of Jiang Gu, M.D., Ph.D.
  Honors Thesis: Discovery of Expression of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in nervous system

Ph.D. candidate in Pathobiology **2011-present**

Johns Hopkins University School of Medicine, Baltimore, MD

- Laboratory of Richard J. Jones, M.D.
  Dissertation: Role of Cytochrome P450 enzymes in the bone marrow microenvironment of acute myeloid leukemia (AML)

EXPERIENCE

Rotating Student **September 2008-July 2009**

Clinical Laboratories, Department of Pathology, Peking University Third Hospital, Beijing, China

- Two to three month rotations in clinical laboratories, including Anatomic Pathology, Chemistry, Microbiology, Immunology and Hematology
- Performed laboratory testing, evaluated and interpreted data as a technologist with entry level competencies

Research Assistant **January 2006-July 2009**

Center of Molecular Morphology, Peking University, Beijing, China

- Discovered the expression of cystic fibrosis transmembrane conductance regulator (CFTR) in human neurons and ganglia that resulted in first author publication
- Designed, validated and optimized RNA probes for *in situ* hybridization (ISH) assay for *CFTR*
- Optimized RT-qPCR on cells from Laser Microdissection, streamlined protocols for the lab
- Collaborated in multiple projects that lead to publications

Research Assistant **April 2010-May 2011**

Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY

- Contributed to discovery of Dynamin-1 is a key protein for the regulation of amyloid generation in Alzheimer's Disease, resulting in publications
- Initiated and developed Morphology techniques in the lab, trained other technicians

Graduate Student **August 2011-present**

Department of Oncology, Johns Hopkins University, Baltimore, MD

- Identified a mechanism of Cytochrome P450 enzymes (CYPs) in drug resistance and its regulation in acute myeloid leukemia (AML), leading to publications and two clinical trials of CYP3A4 and CYP26 targeting drugs in the Johns Hopkins Hospital
- Key contributor to two grant proposals funded by the NIH and Leukemia & Lymphoma Society in 2014

Rotating Student **September, 2014**

Department of Pathology, Johns Hopkins University, Baltimore, MD

- Rotated with Christopher Gocke, M.D., Director of Molecular Pathology
- Experienced diagnostic analyses in molecular diagnostics for hematologic malignancies and solid tumors, including Next-Generation Sequencing (NGS)-based assays

Rotating Student **April, 2016**

Department of Pathology, Johns Hopkins University, Baltimore, MD

- Rotated with James R. Eshleman, M.D., Ph.D., Associate Director of Molecular Diagnostics Lab
- Enhanced diagnostic and interpretive skills, broaden knowledge of current state technologies in molecular diagnosis

Rotating Student **May, 2016**

Department of Pathology, Johns Hopkins University, Baltimore, MD

- Rotated with Yi Ning, M.D., Ph.D., Director of Cytogenetics Lab
- Gained more experience in diagnose common hematologic malignancies and solid tumors by FISH
- Processed BM samples and prepared slides in a collaborative project attempting to perform targeted FISH analysis of CD34+ cells from CLL patients

PEER-REVIEWED PUBLICATIONS

Yong Guo, Min Su, **Meng Su**, Michael A. McNutt, Jiang Gu. Expression and Distribution of Cystic Fibrosis Transmembrane Conductance Regulator in Neurons of the Spinal Cord. *J Neurosci Res* 2009;87(16):3611–9

Yan Zhou, Peng Pan, Lu Yao, **Meng Su**, Ping He, Na Niu, Michael A. McNutt, Jiang Gu. CD117 Positive Cells of the Heart-Progenitor Cells of Mast Cells? *J Histochem Cytochem* 2010;58(4):309-16

Zhengshan Chen, Xinrui Huang, Jiuxiang Ye, Peng Pan, Qi Cao, Baokai Yang, Zhuo Li, **Meng Su**, Chen Huang, Jiang Gu. Immunoglobulin G is present in a wide variety of soft tissue tumors and correlates well with proliferation markers and tumor grades. *Cancer* 2010;116(8):1953-63

Meng Su, Yong Guo, Yingying Zhao, Christine Korteweg, Jiang Gu. Expression of Cystic Fibrosis Transmembrane Conductance Regulator in paracervical ganglia. *Biochem Cell Biol* 2010;88(4):747-55

Li Zhu, **Meng Su,** Louise Lucast, Lijuan Liu, William J Netzer, Samuel E Gandy, Dongming Cai. Dynamin 1 Regulates Amyloid Generation through Modulation of BACE-1. *PLoS ONE* 2012;7(9):e45033

Yu Lei, Tao Huang, **Meng Su**, Jin Luo, Christine Korteweg, Jing Li, Zhengshan Chen, Yamei Qiu, Xingmu Liu, Meiling Yan, Yun Wang, Jiang Gu. Expression and Distribution of Immunoglobulin G in Normal Liver, Hepatocarcinoma and Post Partial-hepatectomy Liver. *Lab Invest* 2014;94(11):1283-95

Jiang Gu, Yu Lei, Yuanping Huang, Yingying Zhao, Jing Li, Tao Huang, Junjun Zhang, Juping Wang, Xiaodong Deng, Zhengshan Chen, Christine Korteweg, Ruishu Deng, Meiling Yan, Qian Xu, Shengnan Dong, Monghong Cai, Lili Luo, Guowei Huang, Yun Wang, Qian Li, Changmei Lin, **Meng Su**, Chunzhang Yang and Zhengping Zhuang. Fab fragment glycosylated immunoglobulin G may play a central role in placental immune evasion. *Human Reproduction* 2015;30(2):380-91

Meng Su, Salvador Alonso, Maureen Kane, Richard J Jones, Gabriel Ghiaur. All-Trans Retinoic Acid Activity in Acute Myeloid Leukemia: Role of Cytochrome P450 Enzyme Expression by the Microenvironment. *PLoS ONE* 2015;10(6): e0127790

Salvador Alonso, **Meng Su**, Richard Jones, Gabriel Ghiaur. Human Bone Marrow Niche Chemoprotection Mediated by Cytochrome P450 Enzymes. *Oncotarget* 2015;6(17):14905-12.

Meng Su, Yu-Ting Chang, Daniela Hernandez, Richard Jones, Gabriel Ghiaur. Chemotherapy drug metabolizing Enzymes are Modulated by the Malignant Clone and Induction Chemtherapy in AML. (Manuscript in preparation)

Daniela Hernandez, Kelly Norsworthy, **Meng Su**, Salvador Alonso, Richard Jones, Gabriel Ghiaur. Overcoming Bone Marrow Stromal Protection from Retinoic Acid Induced Differentiation in APL and Non-APL Cells. (Manuscript in preparation)

CONFERENCE ABSTRACTS

Meng Su, Richard J Jones, Gabriel Ghiaur. Bone Marrow Stromal Expression of Cytochrome P450 Enzymes Protects Acute Myeloid Leukemia From All-Trans Retinoic Acid. *Blood* 2013; 122(21):1449

Salvador Alonso, **Meng Su**, Richard Jones, Gabriel Ghiaur. The stem cell niche detoxifies chemotherapy and protects malignant hematopoietic cells via expression of cytochrome P450 enzymes. *Cancer Research* 2014; 74(19):4842

Salvador Alonso, **Meng Su**, Rick Jones, Gabriel Ghiaur. Cytochrome P450 and drug metabolism in the stem cell niche. *Clinical Lymphoma, Myeloma and Leukemia* Epub August 2015

Kelly J Norsworthy, Daniela Hernandez, **Meng Su**, Megan E McCray, Christopher A Esteb, Roshantha A Chandraratna, B. Douglas Smith, Richard J Jones and Gabriel Ghiaur. RAR-Alpha Targeting Compounds Overcome Bone Marrow (BM) Stromal Protection of AML By CYP26. *Blood* 2015; 126(23):2474

RESEARCH PRESENTATIONS

04/2013	15th Pathology Young Investigator's Day, Johns Hopkins University, Baltimore, MD Poster presentation: "Stromal cells protect AML from retinoic acid via cytochrome P450"
12/2013	55th American Society of Hematology (ASH) Annual Meeting, New Orleans, LA Poster presentation: "Bone Marrow Stromal Expression of Cytochrome P450 Enzymes Protects Acute Myeloid Leukemia from All-Trans Retinoic Acid"
04/2014 CA	105th American Association for Cancer Research (AACR) Annual Meeting, San Diego, Poster contribution: "The stem cell niche detoxifies chemotherapy and protects malignant hematopoietic cells via expression of cytochrome P450 enzymes"
04/2014	16th Pathology Young Investigator's Day, Johns Hopkins University, Baltimore, MD

Poster presentation: "Bone Marrow Stromal Expression of Cytochrome P450 Enzymes Protects Acute Myeloid Leukemia from All-Trans Retinoic Acid"

09/2014 2014 Society of Hematologic Oncology Annual Meeting, Houston, TX
Poster contribution: "Cytochrome P450 and drug metabolism in the stem cell niche"

03/2015 17th Pathology Young Investigator's Day, Johns Hopkins University, Baltimore, MD
Poster presentation: "Bone Marrow Stromal Expression of Cytochrome P450 Enzymes Protects Acute Myeloid Leukemia from All-trans Retinoic Acid"

10/2015 2015 Johns Hopkins Sidney Kimmel Comprehensive Cancer Center Fellow Research Day, Baltimore, MD
Poster contribution: "The Synthethic Retinoid AM80 Overcomes Protection of Acute Myeloid Leukemia by Bone Marrow Stromal CYP26"

12/2015 57th American Society of Hematology (ASH) Annual Meeting, Orlando, FL
Poster contribution: "RAR-Alpha Targeting Compounds Overcome Bone Marrow (BM) Stromal Protection of AML By CYP26"

PEER REVIEW ACTIVITIES

2015 Journal of Experimental & Clinical Cancer Research
The Application of Clinical Genetics
OncoTargets and Therapy

2016 Journal of Translational Medicine
BMC Cancer
Journal of Experimental & Clinical Cancer Research
OncoTargets and Therapy
Journal of Neurological Research and Therapy

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AWARDS AND HONORS

2006 Scholarship for Excellent Student, Peking University

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2008 Best Student Research Award, School of Basic Medical Sciences, Peking University

2012 1st place Research Poster, Pathobiology Retreat, Department of Pathology, Johns Hopkins University

2013 Excellences in Translational Research, 15th Pathology Young Investigator's Day Award, Department of Pathology, Johns Hopkins University

2016 GSA Travel Award, Johns Hopkins University

2016 Chinese Government Award for Outstanding Self-Financed Students Abroad, Chinese Scholarship Council, Ministry of Education of China

CERTIFICATES

12/2015 Genomic Data Science with Galaxy, Johns Hopkins University Bloomberg School of Public Health

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TEACHING AND MENTORING

03/2015 Teaching Assistant, Pathobiology and Disease Mechanisms (Course No. ME:300.710), Johns Hopkins University School of Medicine

2013-2015 Supervised rotating Ph.D. students, new postdoctoral fellows and technicians performing experiments and analyzing results

AFFILIATIONS

2008-2009 Assistant, Chinese Pathologist Association (CPA), Beijing, China

- Surveyed the facts of Department of Pathology of 1100+ hospitals in China in 2009, collected and analyzed the data, composed the keynote presentation for the president of CPA
- Helped organizing two annual conferences attended by 500+ pathologists in 2008 and 2009
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2013- Student Member, American Society of Hematology (ASH)

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